INVESTIGATING THE THERAPEUTIC EFFECTS OF SPHINGOSINE-1-PHOSPHATE AGAINST HUMAN BREAST CANCER *IN VITRO* AND *IN VIVO*

A Thesis Submitted to the College of

Graduate Studies and Research

in Partial Fulfillment of the Requirements

for the Degree of Master of Science

in the Pharmacy Graduate Program

University of Saskatchewan

Saskatoon, Saskatchewan

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ABSTRACT

Breast cancer is the most common malignancy diagnosed among women and is the first cause of neoplastic death in women globally. In the last decade our understanding of breast cancer biology has increased and led to the development of a number of targeted therapies, one of which is targeting the cell apoptosis pathway. One of the new targeting pathways under investigation, which was found to be involved in both cell apoptosis and cell proliferation processes, is the sphingolipid signalling pathway. The sphingolipid pathway represents a group of intracellular and extracellular bioactive lipid molecules, including ceramide, ceramide- 1-phosphate, sphingosine, and sphingosine-1-phosphate (S1P). In my research, I focused on the role S1P plays in breast cancer and its potential application as a therapeutic agent. I examined the effects of S1P on the apoptosis, proliferation, and cytotoxicity of different types of breast cancer cell lines *in vitro*. In addition, I evaluated the effect of both low and high doses of S1P when co-administrated with anticancer drugs commonly used in breast cancer treatment *in vitro* and *in vivo*. Moreover, I studied the S1P cellular distribution following exogenous administration. My results demonstrate that S1P can selectively induce apoptosis in breast cancer cells without harming normal breast cells and that S1P is more effective against aggressive breast cancer cells. Another major finding of my study is that S1P can increase the efficacy of chemotherapies against human breast cancer cells. Although S1P cannot directly substitute the current chemotherapies, S1P may function as a good candidate for combination therapy. Furthermore, my work showed that the pro-apoptotic and anti-proliferative effect of S1P is correlated with its intracellular action and that chronic exposure of exogenous S1P *in vivo* is not toxic to the major organs. Certainly, S1P inclusion in breast cancer treatment modalities may decrease the

morbidity and mortality of breast cancer patients and improve clinical outcomes. Further investigations are required to understand the mechanism by which S1P induces apoptosis and inhibits cell proliferation.

ACKNOWLEDGMENTS

I like to acknowledge the support of my supervisor Dr. Jian Yang, my committee member Dr. Jane Alcorn, and to the staff and faculty of the College of Pharmacy and Nutrition.

DEDICATION

This thesis is dedicated to my parents, Mansour Ahmad Sultan and Nadiah Anbeer Al-bolushi, who always believe in me and support me to achieve my dreams.

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LIST OF ABBREVIATIONS

AA	Arachidonic acid
ABCA1	ATP-binding cassette sub-family A
ABCC1	Multidrug resistance associated protein 1
ABCC7	Cystic fibrosis transmembrane conductance regulator; also known as CFTR
AC	Adenocarcinoma
Apaf-1	Apoptotic Protease-Activating Factor
ATCC	American Type Culture Collection
BAD	Bcl-2-associated death promoter
BAK	Bcl-2 homologous antagonist/killer
Bax	Bcl-2–associated X protein
Bid	BH3 interacting-domain death agonist
BRCA1	Breast cancer type 1 gene
BRCA2	Breast cancer type 2 gene
Ca^{2+}	Calcium ion
cc	Millilitre
CDK	Cyclin dependent kinase
Cer	Ceramide
Cer K	Ceramide kinase
CERT	Ceramide transporter protein

CIAPIN1	Cytokine-induced apoptosis inhibitor 1
cm^2	Centimeter squared
CO_2	Carbone dioxide
conc.	Concentration
COPD	Chronic Obstructive Pulmonary Disease
COX2	Cyclooxygenase enzyme subtype 2
DISC	Death inducing signaling complex
DNA	Deoxyribonucleic acid
EDG	Endothelial differentiation gene
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
ERt	Endoplasmic reticulum
ER	Estrogens receptor
ERK	Extracellular signal-regulated kinase
FADD	Death domain containing protein adaptor
Fas	Tumour necrosis factor super-family 6 or apoptosis antigen 1 (APO-1)
FSH	Follicle stimulation hormone
gm	Grams
HCL	Hydrochloric acid
HER2	Human epidermal growth factor receptor 2
hr	hour

IC_{50}	Half maximum inhibitory concentration
IgE	Immunoglobulin E
IGF-I and II	Insulin-like growth factor I and II
IL-1B	Interleukin-1 beta
JNK	Stress activated protein kinase
KCL	Potassium chloride
KH_2PO_4	Potassium dihydrogen phosphate
KSR	Kinase supressor of Ras
L	Liter
LDH	Lactate dehydrogenase
LH	Luteinizing hormone
MAPK	Mitogen-activated protein kinase
µg	Micrograms
µL	Microliter
µM	Micromolar
mg	Milligrams
mL	Milliliter
mm^3	Cubic milimeter
mM	Millimolar
mTOR	Mammalian target of Rapamycin
Na_2HPO_4	Disodium hydrogen phosphate

NaCL	Sodium chloride
nm	Nanometer
nM	Nanomolar
NSAID	Non steroidal anti-inflammatory drugs
PDGF	Platelet-drive growth factor
PGE2	Prostaglandin E2
P-gp	P-glycoprotein transporter
pH	negative decimal logarithm of the hydrogen ion activity in a solution
PHB2	Prohibitin 2
PI3K/AKT	Phosphatidylinositol-3-kinase/ Serine-threonine protein kinase
PKC	Atypical protein kinase C
PP 1	Phosphoprotein phosphatase 1
PP 2A	Phosphoprotein phospahtase 2A
PR	Progesterone receptor
PRL	Prolactin
S1P	Sphingosine-1-phosphate
S1PRs	Sphingosine-1-phosphate receptors
SD	Standard deviation
SK	Sphingosine kinase
SM	Sphingomylein
SMase	Sphingomylein synthase

Sph	Sphingosine
SPL	Sphingosine-1-phosphate lyase
TGF-α and β	Transforming growth factors alpha and beta
TNF-α	Tumour necrosis factor alpha
TP53	Tumour protein 53
TRADD	Death domain containing protein adaptor
UVB	Ultraviolet B radiation
VEGF	Vascular endothelial growth factor
52K protein	Procathepsin D

1. LITERATURE REVIEW:

1.1. BREAST CANCER

1.1.1. INTRODUCTION

Breast cancer is the most common malignancy diagnosed among women in both developed and developing countries and is the first cause of neoplastic death in women globally. According to global cancer statistics published by the International Agency of Research on cancer (IARC) of the World Health Organization (WHO), breast cancer incidence approximated 1,384,155 cases worldwide in 2008 and the estimated number of deaths in the same year was around 458,503 cases (Figure 1). The causes of breast cancer remain unknown in most patients but studies have found a number of risk factors that contribute to its development (Kumar & Clark, 2002, p. 474). It is well known that breast cancer, like other neoplastic diseases, is a result of a genetic mutation that leads to abnormalities in the cell cycle progression, differentiation, apoptosis, proliferation, growth signalling, DNA repair, and DNA damage (Kumar & Clark, 2002, p. 474). Today, due to advances in molecular biology, a number of molecular pathways that correlate with breast cancer development have been identified, and based on such research, new targeting therapies have been developed to decrease the breast cancer mortality rate and to improve the patient clinical outcomes.

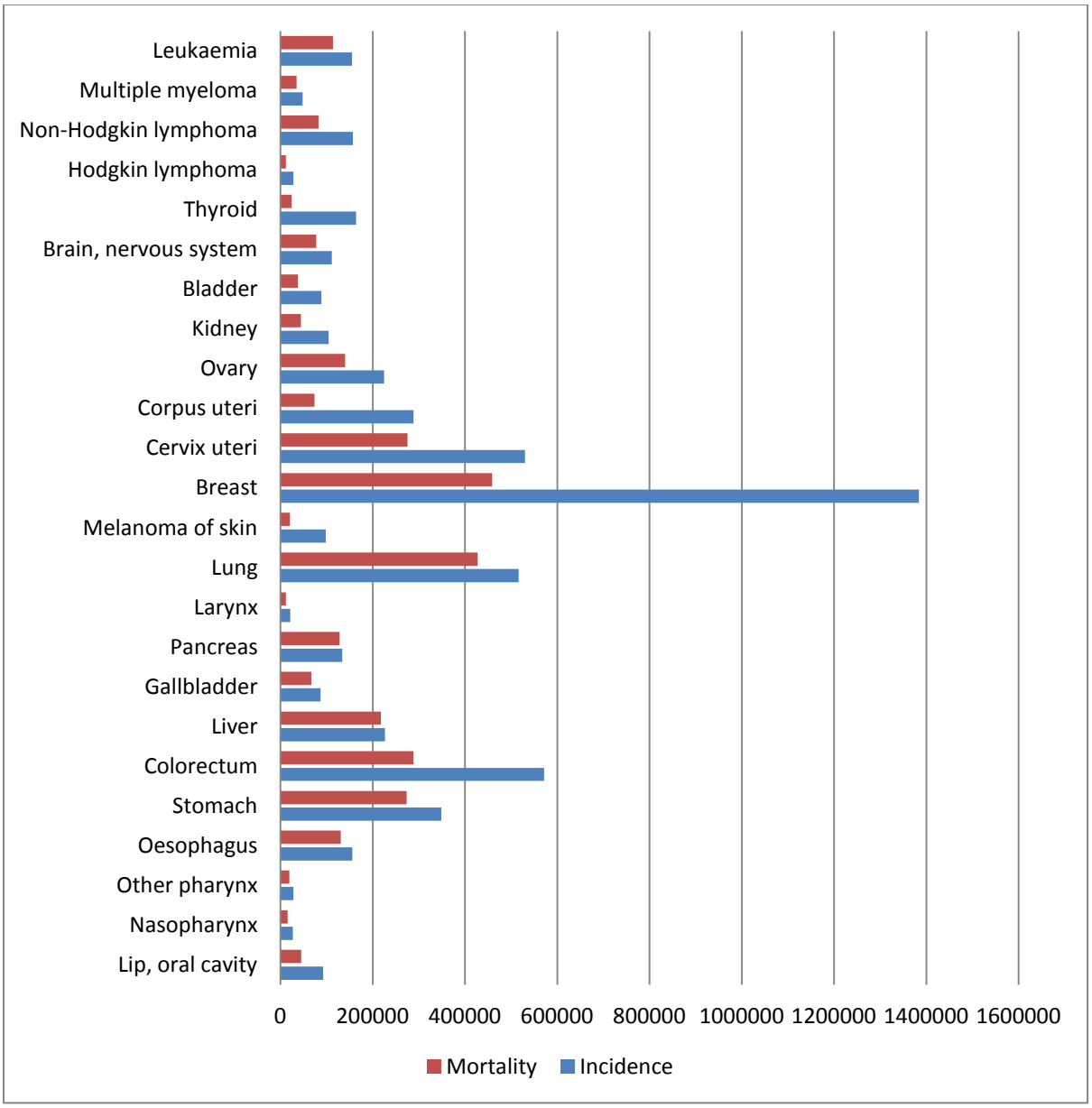


Figure 1. Cancer incidence and mortality rate among women worldwide according to the 2008 global cancer statistics published by International Agency of Research on cancer (IARC) of the World Health Organization (WHO). Breast cancer has the highest incidence rate in women compared to other types of cancer and is the primary cause of death.

1.1.2. ETIOLOGY AND EPIDEMIOLOGY

According to the World Cancer Report of 2008 published by WHO, the incidence of breast cancer is high in North America, South America, Europe and Australia; and the lowest rates are recorded in most countries of sub-Saharan Africa, China, and Eastern Asia, except Japan. These statistics suggest that breast cancer development is associated with environmental factors that vary in different countries and regions. In addition, the variations between Eastern and Western lifestyles and diets could play a major role in breast cancer development. Several risk factors are associated with breast cancer development (Jones & Burris-III, 2000, p.1771). These risk factors can be divided into three categories: environmental, genetic and endocrinal (Jones & Burris-III, 2000, p.1769).

The dietary differences between the East and West, especially related to the consumption of fat, could be a link to the higher incidence of breast cancer in Western countries compared with Eastern countries (Jones & Burris-III, 2000, p.1771). Consumption of a high fat diet will increase the risk of developing breast cancer (Jones & Burris-III, 2000, p.1771). Also, obesity, which is more common in Western countries than Eastern countries, could be a contributor to the higher incidence of breast cancer in Western countries. Obesity increases the estrogen level and causes alterations in glucose metabolism, which in turn increases the risk of developing breast cancer (Jones & Burris-III, 2000, p.1771). In addition, people who smoke tobacco and consume alcohol are at a higher risk of developing breast cancer (Jones & Burris-III, 2000, p.1771).

Family history is an important risk factor for breast cancer development. Women with a first-degree relative (i.e., mother or sister) with breast cancer have a higher probability of developing breast cancer compared with women who do not have relatives with breast cancer (Jones & Burris-III, 2000, p.1769). In addition, women who carry BRCA1 and BRCA2 gene mutation are

at a higher risk and they should be encouraged to educate themselves and practice early detection methods (i.e., self-examination and annual mammography) (Jones & Burris-III, 2000, p.1769). Women who have fibrocystic disease or fibroadenoma of the breast are at higher risk and need close monitoring for breast cancer development (Jones & Burris-III, 2000, p.1770).

Exposure to exogenous hormones (i.e., oral contraceptive or hormonal replacement therapy) will increase the risk for breast cancer development. This is because estrogen promotes the growth of breast cells and significant clinical evidence links estrogen administration and breast cancer development (Jones & Burris-III, 2000, p.1770). In addition, women who have early menarche (i.e., before the age of 12), late menopause (i.e., after the age of 55), or late first full-term pregnancy (i.e., after the age of 35) are at a higher risk (Jones & Burris-III, 2000, p.1770). However, it has been found that pregnancy and lactation could play protective role against breast cancer development (Jones & Burris-III, 2000, p.1770).

1.1.3. BREAST CANCER BIOLOGY

1.1.3.1. ANATOMY OF HUMAN BREAST

Breasts are complex organs composed of skin, connective tissue, fatty tissue, a branching duct system, abundant blood supply, an extensive lymphatic network, nerves and a glandular structure (Lindley & Michaud, 2005, p.2334). However, the three major components are the mammary gland, the connective tissue and the fatty tissue or adipose tissue (McCool, Stone-Candry & Bradford, 1998). The mammary gland is the functional element of the breast; it is composed of lobules and ducts that are lined with epithelial cells, the milk producing cells of the breast (McCool, Stone-Candry & Bradford, 1998). The connective tissue and the adipose tissue are the structural components of the breast; they give the breast its shape (McCool, Stone-Candry & Bradford, 1998). The arterial and venous system supply the breast with blood; the superficial

and the deeper blood vessels are usually connected with channels of lymphatic system (McCool, Stone-Candry & Bradford, 1998). The primary breast lymph is drained initially into the axillary node and the additional lymph will drain at the parasternal node (McCool, Stone-Candry & Bradford, 1998).

At the time of puberty, women's breasts start to develop under the influence of the sex hormones (Jones & Burris-III, 2000, p.1771). However, breast development during puberty is limited and full growth of the breast occurs during the first full-term pregnancy (Jones & Burris-III, 2000, p.1771). The development and function of the breast are controlled by both the pituitary gland and the ovaries (McCool, Stone-Candry & Bradford, 1998). The pituitary gland will release the follicle stimulating hormone (FSH) and the luteinizing hormone (LH) that will stimulate the ovaries to release estrogen and progesterone. These hormones will facilitate breast growth and development. Prolactin hormone (PRL), which is released by the pituitary gland, also plays a role in breast cancer function and development (McCool, Stone-Candry & Bradford, 1998). Breasts have a great variation in the anatomical presentation and physiological development (McCool, Stone-Candry & Bradford, 1998). Healthy female breasts are seldom symmetrical in both shape and size; usually, the left breast is slightly larger than the right breast (McCool, Stone-Candry & Bradford, 1998). At the onset of menses the breast are largest in size and the smallest in day 4 or 7 of the menstrual cycle (McCool, Stone-Candry & Bradford, 1998). The breast change in size and shape occurs also during pregnancy and lactation (McCool, Stone-Candry & Bradford, 1998)

1.1.3.2. BREAST CANCER DEVELOPMENT

Breast cancer may occur whenever breast cells lose control in the mechanism that governs proliferation, differentiation, and apoptosis (Jones & Burris-III, 2000, p.1771). In breast cancer, the proliferation of tumour cells is much higher than apoptosis. The tumour cells' growth will be under the influence of various hormones (i.e., estrogen and progesterone), oncogenes, and growth factors (Jones & Burris-III, 2000, p.1771-1772). In fact, numerous growth factors play a role in breast cancer cell development, including transforming growth factor alpha and beta (TGF-α and TGF-β), insulin-like growth factors I and II (IGF-I and IGF-II), platelet-derived growth factor (PDGF), and procathepsin D (52K protein) (Jones & Burris-III, 2000, p.1772). Furthermore, estrogen receptors (ER) are highly expressed in most breast cancer cells, so circulating estrogen will promote the growth of breast tumours. Bcl-2 oncogene and tumour suppress p53 gene, which regulates the cell apoptosis, show mutations in many breast cancer patients and promote cancer cell growth (Lindley & Michaud, 2005, p.2339).

1.1.3.3. MOLECULAR BREAST CANCER SUBTYPE:

In the last two decades, our understanding of breast cancer biology has increased. According to this knowledge, we can categorize breast cancer into three sub-types: Luminal/ER positive, HER-2, and Basal (Atieh & Vahdat, 2008, p.310; Davies & Hiscov, 2011). Luminal/ER positive is further sub-divided into: luminal A, which is highly expressed with ER, and luminal B, which is characterized by moderate to low ER expression. HER-2 sub-types are highly expressed with human epidermal growth factor receptors-2 (HER-2). Basal-like or triple negative sub-type is associated with the absence of receptor expression (i.e., ER-ve, PR-ve, and HER-2 -ve) (Davies & Hiscov, 2011). Understanding breast cancer sub-types in each patient is an important prognostic factor as it predicts the clinical outcome, progression, and survival (Atieh & Vahdat,

2008, p.312-313; Davies & Hiscov, 2011). In addition, it is a critical distinction determining the treatment decision by the clinical team (Davies & Hiscov, 2011).

1.1.4. TREATMENT

Different types of therapy are available for the treatment of breast cancer. Some are currently applied and approved for clinical use and others are still in clinical trial. The medical team designs treatment based on the patient's prognostic factors, such as the presence of lymphatic or vascular invasion, tumour size, hormonal receptors status (i.e. ER+, ER-, PR+, or PR-), proliferation index, the expression of HER-2, mutation of tumour suppresser p53 gene, growth factors levels, cathepsin D, and angiogenesis factors (Lindley & Michaud, 2005, p.2338-2340). The treatment options available are as follow: surgery, radiation, chemotherapies, endocrine therapies, and biological therapies (Figure 2). Surgery can include lumpectomy, or simple, or total mastectomy. The lumpectomy is usually followed with radiation therapies and is less invasive than a mastectomy (Lindley & Michaud, 2005, p.2341). However, mastectomy is a good option to ensure no recurrence of breast cancer (Lindley & Michaud, 2005, p.2341).

Chemotherapies are mainly used in combination. The most common chemotherapies used in breast cancer are cyclophosphamide, doxorubicin, 5-flurouracil, docetaxel, paclitaxel, methotrexate, and vincristine (Lindley & Michaud, 2005, p.2355-2357). Endocrine therapies involve the use of anti-estrogen agents, like tamoxifen or aromatase inhibitors like letrozole (Lindley & Michaud, 2005, p.2350-2355). Biological therapies include the monoclonal antibodies (e.g.,trastuzumab) and tyrosine kinase inhibitors (e.g., lapatinib) (Lindley & Michaud, 2005, p.2357-2358). Despite the improvement in the clinical outcome of the breast cancer patient through the use of these treatment options, the mortality and morbidity rate of breast cancer remains high. We need to find a new target to treat breast cancer, such as targeting the

sphingolipid signalling pathway that has been found to be involved in cell apoptosis and proliferation processes in both normal and cancer cells.

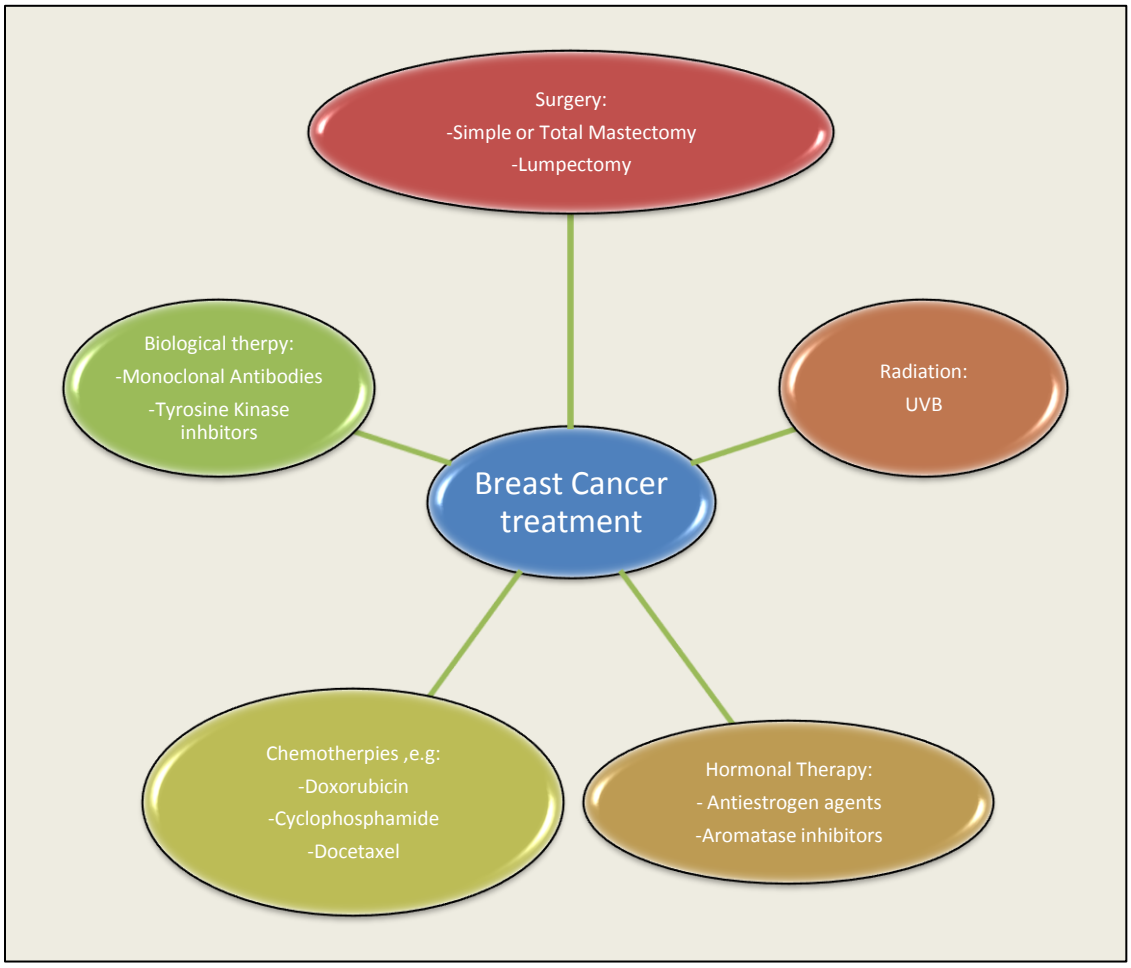


Figure 2. Curruent breast cancer treatment options.

1.2. SPHINGOLIPID SIGNALLING PATHWAY

1.2.1. INTRODUCTION

The sphingolipid signalling pathway represents a group of intracellular and extracellular bioactive lipid molecules, including ceramide (Cer), ceramide-1-phosphate, sphingosine (Sph), and sphingosine-1-phosphate (S1P) (Hait et al., 2006; Segui et al., 2006). Studies have shown that sphingolipids play an important role in regulating cell growth and survival; additional physiological functions include cell motility, differentiation, stress response, protein synthesis, carbohydrate metabolism, angiogenesis, and immunity (Hait et al., 2006; Segui et al., 2006; Sabbadini, 2006). Any alteration on the sphingolipid signalling will result in the development of serious pathophysiological disorders, such as cancer, cardiovascular diseases, diabetes, asthma, and inflammatory and infectious diseases (Hait et al., 2006; Segui et al., 2006; Sabbadini, 2006) (Figure 3). Subsequent sections are focused on the role that sphingolipids play in breast cancer development, progression, and drug resistance development.

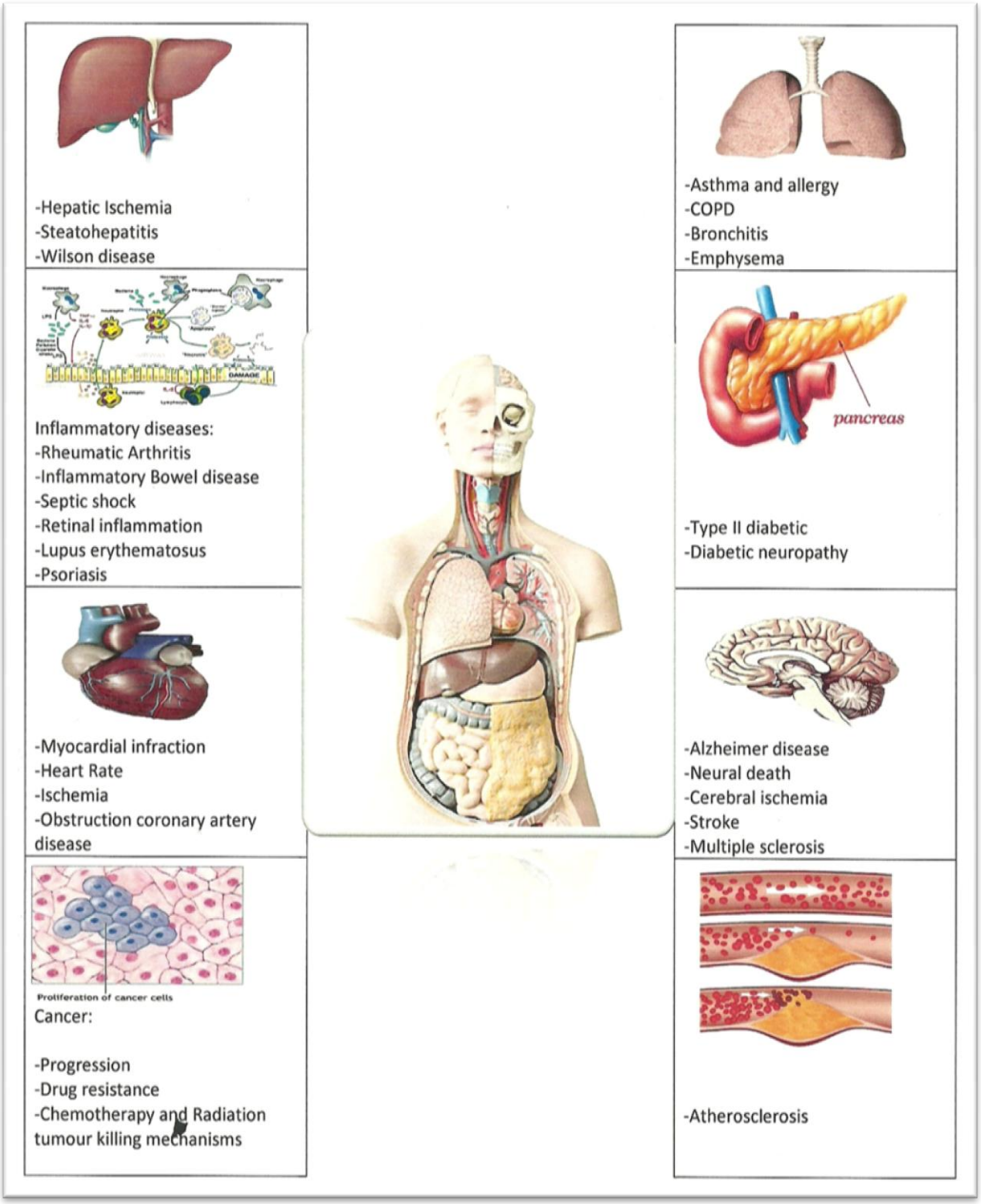


Figure 3. Diseases associated with alteration in the sphingolipid pathway. Sphingolipids affect almost every organ in the human body and are associated with numerous pathophysiological disorders.

1.2.2. SPHINGOLIPID CHEMICAL STRUCTURE

Sphingolipids are a class of lipids that are defined by their eighteen carbon amino-alcohol backbones (Gault, Obeid & Hannun, 2010). The modification of this basic structure will give rise to a wide family of sphingolipids (Gault, Obeid & Hannun, 2010). Sphingosine, phytosphingosine and dihydrosphingosine act as the precursors upon which more complex sphingolipids are formed by phosphorylation or acetylation (Gault, Obeid & Hannun, 2010).

1.2.3. ROUTE OF SYNTHESIS

Sphingolipids are generated by two routes: *de novo* biosynthesis and recycling of sphingomyelin (SM) (Figure 4). SM is one of the membrane phospholipids found in the plasma membrane, nucleus, endoplasmic reticulum (ERt), Golgi apparatus, and lysosome; however, 70-90% of SM is present mainly in the plasma membrane (Gault, Obeid & Hannun, 2010). The *de novo* biosynthesis occurs in the ERt, which contains the enzymes responsible for the biosynthesis of ceramide (Cer) (Gault, Obeid & Hannun, 2010). The cytosolic serine and palmitoyl-CoA molecules are condensed in the ERt by the action of three enzymes serine palmitoyltransferase, 3-ketodihydrosphingosine reductase, and dihydroceramide synthase to give dihydroceramide, which then desaturates to give Cer, the first sphingolipid present in the cycle (Gault, Obeid & Hannun, 2010). The Cer generated by this route will travel from ERt to Golgi apparatus by CERT (ceramide transporter protein) where it converts to SM, glycosphingolipids, or Cer-1-Phosphate (Gault, Obeid & Hannun, 2010). The recycling of the SM route occurs as a result of SM catabolism that will produce Cer, Sph, and S1P by the action of the following enzymes: SMase, ceramidase, and SK, respectively (Gault, Obeid & Hannun, 2010). S1P is the last sphingolipid in the cycle and it will reversibly undergo dephosphorylation *via* S1P-phosphatase

to form Sph, or it will undergo an irreversible cleavage *via* S1P-lyase to form hexadecenal and ethanolamine phosphate (Gault, Obeid & Hannun, 2010).

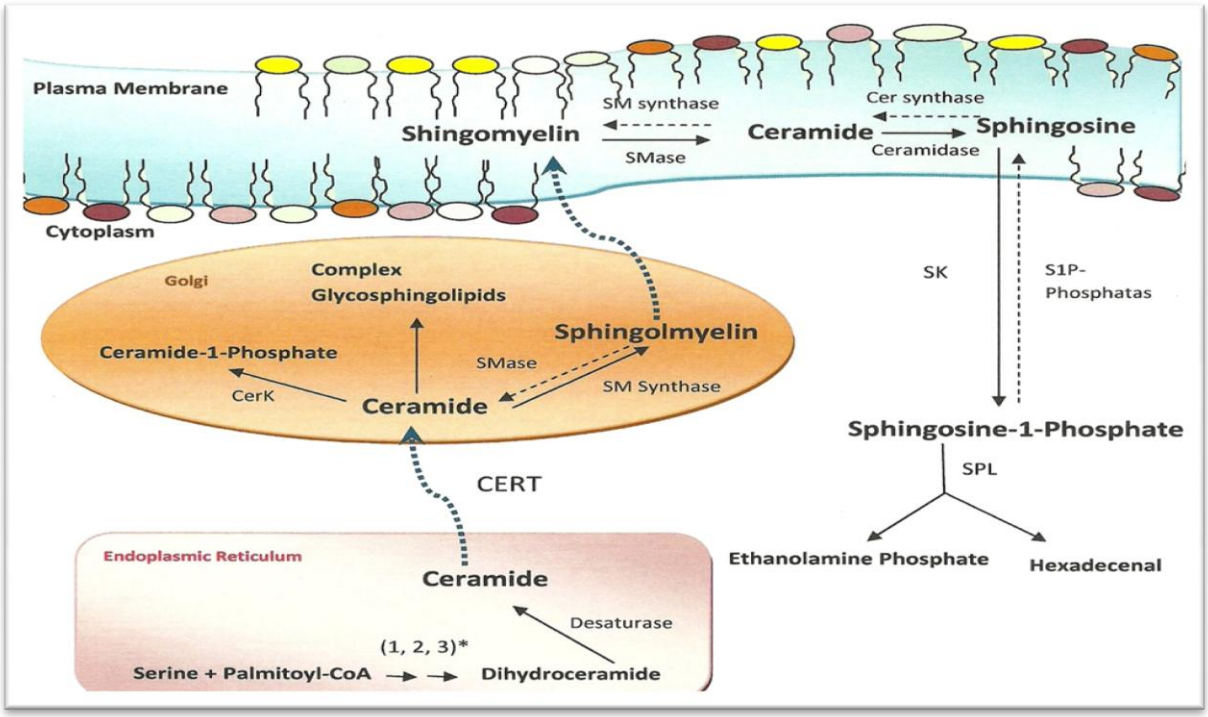


Figure 4. Sphingolipid metabolism: The *de novo* biosynthesis pathway and the recycling of sphingomyelin. The first step begins in the endoplasmic reticulum were serine and palmitoyl-CoA condense by the action of three enzymes to generate dihydroceramide that is desaturated to give rise to ceramide. The ceramide produced in the ERt will traffic to Golgi apparatus by CERT (ceramide trasporter protein) where it is further metabolised to sphingomyelin by shingomyeline synthase, glycosphingolipids, or phosphorylate by ceramide kinase to give ceramide-1-phosphate. The recycling of sphingomyelin in the plasma membrane, lysosome or nucleus by sphingomyelinase enzyme will generate ceramide that is further degraded to sphingosine by the action of ceramidase. Sphingosine will be phosphorylated by sphingosine kinase to give sphingosine-1-phosphate, the last sphingolipid in this cycle that will undergo irreversible cleavage by the action of sphingosine-1-phosphate lyase to ethanolamine phosphate and hexadecenal or dephosphorylation by sphingosine-1-phosphate phosphatase to turn back to sphingosine.

*(1, 2, and 3): serine palmitoyltransferase, 3-ketodihydrosphingosine reductase, and dihydroceramide synthases.

- This figure is adapted from, (Gault, Obeid and Hannun, 2010).

1.2.4. SPHINGOLIPID AND CELL REGULATION

The sphingolipid signalling pathway is important for cell viability. Studies in yeast culture have shown that cells cannot survive when they are unable to produce sphingomyelin (Cerantola et al., 2009) or if they have a defect in the *de novo* synthesis route (Sutterwala et al., 2007). In addition, evidence shows that the balance between Cer- Sph and S1P levels is critical to the cells; it determines the cell's fate towards death or survival (Oskouian & Saba, 2010; Sabbadini, 2006). Many studies suggest that Cer and Sph are the death-promoting sphingolipids, and S1P is the growth-promoting sphingolipid (Oskouian & Saba, 2010; Sabbadini, 2006). When a cell undergoes apoptosis, it directs the sphingolipid signalling pathway towards Cer and Sph synthesis; in contrast, when it undergoes proliferation it will direct this pathway toward S1P synthesis (Oskouian & Saba, 2010; Sabbadini, 2006). Sphingolipids affect every aspect involved in cell regulation (Zheng et al., 2006).

1.2.4.1. CELL APOPTOSIS PATHWAY

Apoptosis, also called "cellular suicide", is the programmed death of the cell. It is different from necrosis in that it occurs under a normal physiological condition (Makin, 2002). There are two pathways in which a cell undergoes apoptosis: the extrinsic route and the intrinsic route. The extrinsic pathway is mediated by the activation of the death receptors that are present in the cell surface (i.e. TNF-α and Fas receptors) (Makin, 2002). The stimulation of these receptors activates caspase-8, causing activation of downstream caspases and apoptosis (Makin, 2002). The intrinsic pathway is mediated through permeabilization of the outer mitochondrial membrane, which leads to the release of mitochondrial cytochrome c, the activation of caspase-9, and, as a result, apoptosis (Makin, 2002). This pathway is mediated *via* the activation of the pro-apoptotic members of the Bcl-2 protein family, which are present in the mitochondrial surface.

These members include: Bcl-x(s), BAX, BAK, BAD, and BID (Makin, 2002). However, this route can be blocked by the activation of anti-apoptotic members of Bcl-2 protein family, such as Bcl-2 and Bcl-x (L) (Makin, 2002). Sphingolipids have been found to play a major role in regulating both apoptosis routes (Oskouian & Saba, 2010).While Cer will enhance apoptosis, S1P will protect the cell against apoptosis and will direct the cells towards proliferation and survival by the activation of sphingosine-1-phosphate receptor subtype 1 ($S1PR_1$) and sphingosine-1-phosphate receptor subtype 3 ($SIPR_3$). At the same time, S1P could also enhance apoptosis by unknown mechanisms (Oskouian & Saba, 2010).

Cer exerts its apoptotic effect through the activation of the intrinsic and extrinsic apoptosis pathways (Oskouian & Saba, 2010) (Figure 5). It activates the extrinsic pathway though stimulation of both death receptors (i.e., TNF-α and Fas) (Oskouian & Saba, 2010). Cer activates TNF-α receptor *via* regulating Bid. It directly binds to cathepsine D, which cleaves Bid, causing activation of TNF-α. Cer can also stimulate the Fas receptor through its biophysical properties. Cer aggregates into the surface membrane, allowing the membrane to undergo structural changes that causes the activation of Fas (Zheng et al., 2006; Oskouian & Saba, 2010). Cer affects the intrinsic pathway by regulating two members of Bcl-2 protein family: the pro-apoptotic protein Bax and the anti-apoptotic Bcl-2 protein (Oskouian & Saba, 2010). Cer can down-regulate Bcl-x(L), stimulate Bcl-x(s), and activate Bax causing induction of apoptosis.

Conversely, S1P may stimulate cell proliferation and inhibit cell apoptosis by activating a group of five cell surface receptors that are a family of G-protein-coupled receptors (i.e., S1PRs) (Oskouian & Saba, 2010). The activation of S1PRs, particularly, $S1PR_1$ and $S1PR_3$, blocks the apoptosis pathway by down-regulating Bax expression, inactivating Bad, inhibiting any change in the mitochondrial membrane potential, preventing cytochrome c release, and protecting

against Fas induced apoptosis (Oskouian & Saba, 2010) (Figure 5). Oddly, evidence suggests that S1P can induce apoptosis independent of S1PRs, and while the mechanism is still unclear, some researchers correlate S1P apoptotic effect to its intracellular action (Oskouian & Saba, 2010).

1.2.4.2. DEATH SIGNALLING

Cer has an effect either directly or indirectly on different pathways that promote cell death (Signorelli & Ghidoni, 2005; Zheng et al., 2006) (Figure 6). It can induce the stress activating protein kinase (JNK), and phosphoprotein phosphatease 1 and 2A (PP1 and PP2A) and activate kinase suppressor of Ras (KSR) and the pro-apoptotic atypical protein kinase C (PKC) causing cell death (Signorelli & Ghidoni, 2005; Zheng et al., 2006). Contrarily, S1P will oppose Cer actions by suppressing JNK, leading to cell survival and growth; it can also increase the levels of pro-growth and angiogenesis factors such as EGF, PDGF, and VEGF (Oskouian & Saba, 2010).

1.2.4.3. GROWTH AND SURVIVOR SIGNALLING

Stimulation of the PI3K/AKT pathway will facilitate the cell cycle progression, inhibit cell apoptosis, suppress autophagy, and enhance nutritient uptake and cell growth and survival (Oskouian & Saba, 2010). Studies have found in many cancer patients the presence of a mutation in this pathway, which is usually correlated with tumour progression and treatment resistance (Oskouian & Saba, 2010). Cer acts as a down-regulator of the PI3K/AKT pathway causing inhibition of the cell proliferation and induction of autophagy (Oskouian & Saba, 2010). On the other hand, S1P acts as an up-regulator for the PI3K/AKT pathway causing stimulation of cell proliferation and increases cell survival; this effect requires the activation of $S1PR_1$ and $S1PR_3$ (Oskouian & Saba, 2010). Surprisingly, S1P activation of $S1PR_2$ blocks the PI3K/AKT signalling pathway. In addition, sphingolipids have been found to affect another survival

signalling pathway the ERK/MAPK pathway (Oskouian & Saba, 2010). S1P will activate this pathway, leading to cell survival and protection against cell death (Oskouian & Saba, 2010).

1.2.4.4. CELL MOTILITY

Cells rely on cellular movement to maintain some important biological processes, such as cellular development and response to infection and injury. Growth factors are important regulators of the cell movement mechanism. Generally, S1P can stimulate cell motility through the activation of S1PRs surface receptors, particularly $S1PR_1$ and $S1PR_3$ (Oskouian & Saba, 2010; Pyne & Pyne, 2010). It has been found that EGF stimulates SK to increase the production of S1P, which will act on $S1PR_1$ and $S1PR_3$ to induce cell motility. Oddly, the activation of $S1PR_2$ inhibits cell motility. As a result, the S1P effect on cell motility will be determined by the S1PRs sub-type predominantly expressed in certain tissues (Oskouian & Saba, 2010; Pyne & Pyne, 2010).

1.2.4.5. CELL CYCLE PROGRESSION

The cell cycle involves DNA replication and cell division and has four phases known as G1, S, G2 and M phase (Vermeulen, Van Bockstaele & Berneman, 2003). The cyclins and the cyclin dependent kinases (CDK) are the two key regulatory proteins of the cell cycle (Vermeulen, Van Bockstaele & Berneman, 2003). The G0 is known as the resting phase, in which the cell leaves the cycle and ceases replication (Vermeulen, Van Bockstaele & Berneman, 2003). The G1, S, and G2 phases are the interphase, during which cell growth takes place and DNA replication occurs (Vermeulen, Van Bockstaele & Berneman, 2003). The M phase is the mitotic phase, during which the cell splits itself into two daughter cells (Vermeulen, Van Bockstaele & Berneman, 2003). The CDKs are activated upon binding with cyclins and different CDK-cyclin complexes will affect different phases of the cell cycle (Vermeulen, Van Bockstaele &

Berneman, 2003). The most common cyclins include cyclin A, B, C, E, F, G, H, K, and T (Vermeulen, Van Bockstaele & Berneman, 2003). The CDKs which are particularly important in the cell cycle include CDK1, CDK2, CDK4, and CDK6 (Vermeulen, Van Bockstaele & Berneman, 2003). Cer may promote cell death through inhibition of the cell cycle progression (Oskouian & Saba, 2010; Lee, Bielawska & Obeid, 2000). Cer can cause G1 cell cycle arrest *via* regulation of CDK2-cyclin complex activity and induction of p21 expression that activates p53 leading to inhibition of the DNA replication (Oskouian & Saba, 2010; Lee, Bielawska & Obeid, 2000). In addition, Cer can inhibit telomerase expression and this will block cell growth because telomerase is a ribonucleoprotein enzyme complex that is required during the replication phase of the cell cycle and its inhibition will interrupt the cell growth (Oskouian & Saba, 2010). Therefore, Cer causes cell death through suppression of CDK2-cyclin complex activity and inhibition of telomerase enzyme.

1.2.4.6. CELL AUTOPHAGY

Autophagy is a catabolic pathway, which involves the degradation of unnecessary or dysfunctional cellular components by lysosomes (Janku, McConkey, Hong & Kurzrock, 2011). It is a tightly controlled process, which helps to maintain the balance between cell synthesis and degradation (Janku, McConkey, Hong & Kurzrock, 2011). In addition, autophagy is important in maintaining cellular homeostasis (Janku, McConkey, Hong & Kurzrock, 2011). Beclin-1 is one of the essential regulators of this process (Chen & Karantza-Wadsworth, 2009). When the cell is suffering from nutrition deprivation it induces the autophagy process. This facilitates the binding of beclin-1 to Bcl-2 pro-apoptotic members, such as BID, BAD, BAK, and BIM causing cell death (Chen & Karantza-Wadsworth, 2009). In addition, the growth factor signalling pathway, PI3K/Akt/mTOR, plays a major role in regulating the autophagy process (Chen & Karantza-

Wadsworth, 2009). Stimulation of the PI3K/Akt/mTOR pathway negatively regulates cell autophagy process (Chen & Karantza-Wadsworth, 2009). Sphingolipids are also involved in regulating the autophagy process (Oskouian & Saba, 2010). Both Cer and S1P cause induction of the cell autophagy but by different mechanisms. Cer induces autophagy by up-regulating beclin-1 and down-regulating the PI3K/AKT pathway, while S1P enhances autophagy independently of the PI3K/AKI pathway and, rather, exerts its effect by direct inhibition of mTOR (Oskouian & Saba, 2010).

1.2.4.7. INFLAMMATION AND IMMUNITY

When cells get infected or injured the inflammatory process will be the first response of the immune system (Aggarwal & Gehlot, 2009). Inflammation is associated with redness and swelling of the injured or infective site (Aggarwal & Gehlot, 2009). The most common regulators of inflammation are the cytokines and eicosanoids. The main cytokines involved in the inflammation are the interleukins, chemokines, and interferons (Aggarwal & Gehlot, 2009). The eicosanoids include prostaglandin and leukotriene. The release of cytokines and eicosanoids promote the movement of leukocytes and cause vasodilatation of the blood vessel (Aggarwal & Gehlot, 2009). Growth factors and cytotoxic factors play a role in regulation of the inflammation response (Aggarwal & Gehlot, 2009). However, studies have found an association between inflammation and sphingolipids, particularly SK-S1P pathway. The SK activity increases in response to inflammatory mediators, such as TNF-α (Pyne & Pyne, 2010). In addition, S1P up-regulates the expression of cyclooxygenase 2 (COX2) and stimulates the production of prostaglandin E2 (PGE2) (Pyne & Pyne, 2010). Moreover, stimulation of $S1PR_1$ enhances immune cell function and plays an important role in controlling lymphocyte recirculation and

trafficking (Hait et al, 2006). Thus, the SK-S1P signalling promotes carcinogenic inflammatory effect *via* generation of PGE2 and up-regulation of COX2 (Oskouian & Saba, 2010).

To conclude, sphingolipid effects on the cells are complex. They are involved in every aspect that regulates cell function and development. Cer and S1P have opposite effects on cell fate; however, their effect on cell autophagy is similar. The S1P function on the cell is much more complex than the other sphingolipids; S1P has a wide range of effects on cell fate and migration (Oskouian & Saba, 2010).

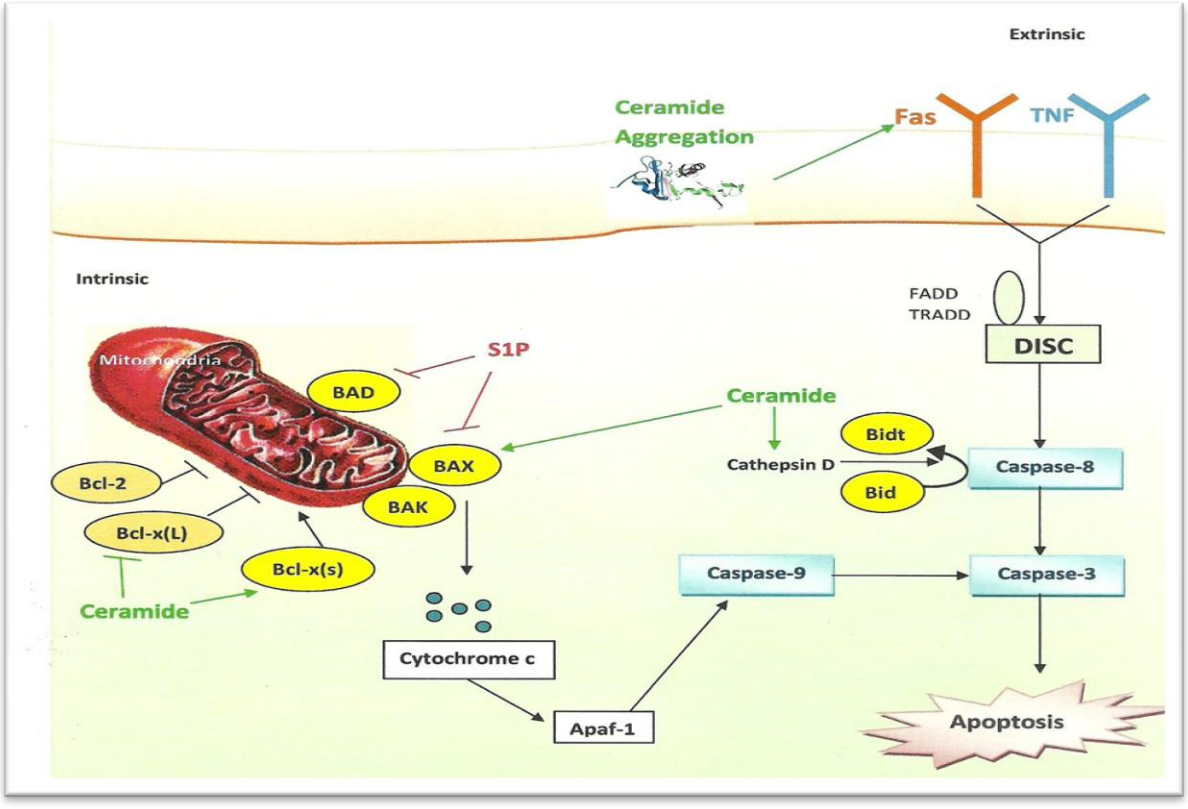


Figure 5. Ceramide and sphingosine-1-phosphate affect on the intrinsic and extrinsic apoptosis pathways. Ceramide will down-regulate the anti-apoptotic protein Bcl-x(L), enhance pro-apoptotic proteins Bcl-x(s) and Bax, stimulate cathepsin D that can activate the pro-apoptotic protein Bid leading to stimulation of TNF-α, and ceramide can aggregate into the membrane causing activation of Fas receptors. In contrast, sphingosine-1-phosphate will down-regulate Bax and Bad protecting the cells against apoptosis.

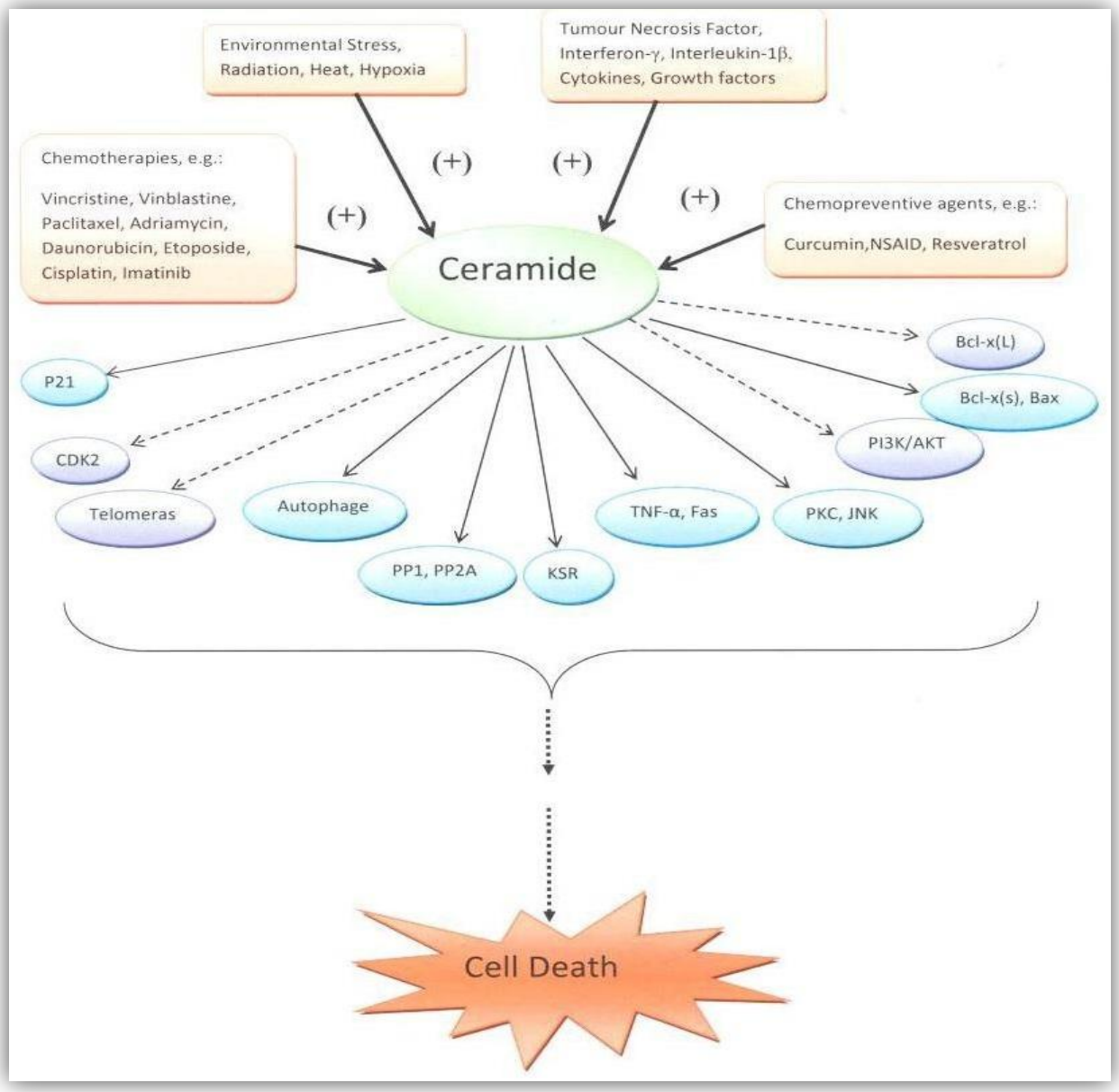


Figure 6. Ceramide stimuli and effect on the cell. Ceramide has diverse stimuli to enhance its production including: environmental stress, TNF, IL-1β, radiation, chemotherapies and chemopreventive agents. The production of ceramide will direct the cell toward death. Ceramide will enhance cell apoptosis by affecting both the intrinsic and the extrinsic pathway, it will arrest cell cycle at G0/G1, and will stimulate different death signals e.g. JNK, KSR, PP1, and PP2A. In addition, it will cause downregulation of PI3K/AKT pathway and will enhance auto-phagy. Note: (→) means stimulation, and (⇢) means inhibition.

1.2.5. SPHINGOLIPIDS AND DISEASES

Any alteration in the sphingolipid signalling pathway is associated with the development of numerous diseases, such as cardiovascular disorders, atherosclerosis, respiratory disorders, diabetes, Alzheimer's, inflammatory diseases, and cancer (Figure 3). For this reason, research in this area has gained high interest among scientists in the last decade. Each sphingolipid has a specific role in cells and regulates important physiological functions. The accumulation of Cer in the brain is associated with cerebral ischemia and stroke; in addition, studies suggest that Cer contributes in the development of Alzheimer's disease (Stiban, Tidhar & Futerman, 2010). Moreover, the up-regulation of Cer *via* the *de novo* pathway is involved in lung alveolar cell apoptosis and contributes to the development of asthma and COPD (Stiban, Tidhar & Futerman, 2010). In addition, the S1P level is elevated in broncholavage fluid of asthmatic patients and many studies suggest that S1P is involved in promoting allergic and inflammatory responses in COPD and asthma patients (Hait et al., 2006). In the last decade remarkable progress has been made to elucidate the involvement of sphingolipids in different disorders and the subsequent sections focus on sphingolipids' role in cancer.

1.2.6. SPHINGOLIPDS AND CANCER

In cancer, studies have shown that sphingolipids and the enzymes that mediate their function and metabolism play a major role in the pathophysiology, progression, and treatment resistance of the disease (Signorelli & Ghidoni, 2005). Sphingolipids are also involved in chemotherapies and the radiation mechanism of action of cancer cells killing and are associated with development of cancer drug resistance. Moreover, sphingolipids regulate cancer metastasis and angiogenesis. Many drugs have been developed to target sphingolipids signalling pathways in cancer treatment.

1.2.6.1. EXPRESSION OF SPHINGOLIPIDS SIGNALLING IN CANCER

Up-regulation of the Cer synthesis enzyme activity will induce apoptosis and its down-regulation will increase tumour growth and survival (Oskouian & Saba, 2010; Signorelli & Ghidoni, 2005). In addition, the ceramidase has been found to be over-expressed in tumour cells and this enhances Cer degradation and protects tumour cells from apoptosis (Oskouian & Saba, 2010). Another enzyme found to play a role in cancer is SK1, which is responsible for the phosphorylation of Sph to generate S1P. SK1 will promote proliferation and survival of tumour cells and its over-expression is correlated with resistance to medications (Pchejetski et al, 2005). SK1 was found to be over-expressed in solid tumours, including breast, kidney, ovary, prostate, and stomach cancers, and this suggests SK1 is involved in breast cancer development and progression (Nagahashi et al., 2012; Olivera et al., 1999).

1.2.6.2. SPHINGOLIPIDIS AS MEDIATORS OF CHEMOTHERAPIES AND RADIATION

Sphingolipids have been found to play role in the radiation, chemotherapy, and chemopreventive agent mechanism of action. Curcumin is a potent chemopreventive agent and studies reveal that it increases Cer levels in colon cancer cells through induction of the *de novo* pathway as part of its mechanism of protecting against cancer development (Moussavi, Assi, Gomez-Munoz & Salh, 2006). The NSAIDs are useful chemopreventive agents and their tumour suppression is related to the elevation of prostaglandin precursor arachidonic acid (AA) that in turn stimulates the conversion of SM to Cer (Chan, Morin, Vogelstein & Kinzler, 1998). Resveratrol is another chemopreventive agent, which exerts its effect by stimulation of the *de novo* synthesis and recycling of SM to increase the level of Cer (Signorlli & Ghidoni, 2005; Scarlatti et al., 2003). Docetaxel is anticancer drug commonly used in breast cancer and induces potent inhibition of SK1 expression and elevates the levels of Cer/S1P ratio as part of its

mechanism of killing cancer cells (Pchejetski et al., 2005). Studies of doxorubicin effects in human head and neck squamous cell carcinoma have found that doxorubicin significantly elevates Cer levels (about 7-fold compared with the control) (Cuvillier et al., 2001; Sankala et al., 2007). Many anticancer medications, such as etoposide (Perry et al., 1999; Sawada et al., 2000), Cisplatin (Min et al., 2007; Noda et al., 2001), imatinib (Bonhoure et al., 2008; Baran et al., 2007), and daunorubicin (Bose et al., 1995) increase the level of Cer as part of their mechanism of killing tumour cells *via* up-regulation of ceramide synthesis enzyme through stimulation of the *de novo* pathway, or *via* activation of SMase enzyme, which increases turnover of SM to Cer. Moreover, UVB radiation increases Cer levels as part of its mechanism of action (Bruno et al., 1998; Pena, Fuks & Kolesnick, 2000; Zeidan et al., 2008; Sakakura et al., 1996; Santana et al., 1996).

1.2.6.3. SPHINGOLIPIDS AND CANCER METASTASIS AND ANGIOGENESIS

As mentioned previously, S1P can regulate cell motility through activation of S1PRs cell surface receptors in both normal and cancer cells. Activation of $S1PR_1$ and $S1PR_3$ will enhance cell motility, while activation of $S1PR_2$ will cause the opposite effect (Oskouian & Saba, 2010). Cancer cell migration increases a tumour's metastatic potential; S1P will increase the risk of cancer metastasis development when the tumour cells highly express with $S1PR_1$ and $S1PR_3$ (Oskouian & Saba, 2010). SK1 stimulation of EGF will trigger cell migration and metastasis (Huwiler & Zangemeister-Wittke, 2007). Moreover, S1P promotes endothelial growth and enhances blood vessel formation by interaction with VEGF signalling (Alvarez, Milstien & Spiegel, 2007). All of these data indicate that SK-S1P pathway may contribute in cancer cell metastasis, angiogenesis, invasiveness and progression.

1.2.6.4. SPHINGOLIPIDS AND CANCER DRUG RESISTANCE DEVELOPMENT

Sphingolipid expression on cancer cells can be correlated with tumour cells sensitivity or resistance to treatment (Oskouian & Saba, 2010). Drug-resistant cells exhibit greater SK1 activity, failure to accumulate Cer, and a higher S1P: Cer ratio (Oskouian & Saba, 2010). The expression of CERT, which is responsible of the transportation of Cer from ERt to the Golgi apparatus, is elevated in tumour-resistant cells (Oskouian & Saba, 2010). Over-expression of SK1 can enhance the expression of the P-glycoprotein (P-gp) transporter. The regulation of P-gp represents a major obstacle in cancer treatment (Huwiler & Zangemeister-Wittke, 2007). The up-regulation of P-gp expression is correlated with chemotherapy failure because P-gp is an efflux transporter that will prevent anti-cancer drugs from entering the tumour cell to exert their effects (Huwiler & Zangemeister-Wittke, 2007).

1.2.6.5. THE MOST COMMON APPROACH USED IN TARGETING SPHINGOLIPID IN CANCER THERAPY

Based on the understanding of cancer biology and the sphingolipid signalling pathway involvement in cancer, a number of new cancer medications that are targeting the sphingolipid pathway have been developed (Huwiler & Zangemeister-Wittke, 2007). These medications promise to decrease the morbidity and mortality rates of women with breast cancer. Some of the strategies used to target sphingolipids include production of synthetic Cer analogs, inhibition of the enzymes that catalyze Cer catabolism, inhibition of SK1, production of S1PRs's antagonist or agonist, and reactivation of genes such as S1P-lyase, SIP-phosphatase, SMase, or dihydroceramide desaturase (Oskouian & Saba, 2010).

1.3. SPHINGOSINE-1-PHOSPHATE

1.3.1. INTRODUCTION

S1P is one of the bioactive sphingolipid metabolites that play an important role in many physiological and pathophysiological processes including regulation of cell growth, angiogenesis, and migration in both normal and tumour cells (Maceyke, Harikumar, Milstien & Spiegel, 2012). S1P has a complex effect on the cell, as it can enhance proliferation, survival, and migration in some cases, but also shows the opposite effects of inducing cell apoptosis and blocking cell movement. Consequently, S1P could play a major role in cancer treatment since it can act on both apoptosis and proliferation. Indeed, many studies found a direct link between S1P and many types of cancer including breast cancer (Signorelli & Ghidoni, 2005a). In the last several years, remarkable progress has been made to elucidate the function and role of S1P in cells. In the following sections, S1P's function, enzymes regulating its level, and its role in breast cancer treatment and prevention will be explained.

1.3.2. THE ENZYMES THAT REGULATE SPHINGOSINE-1-PHOSPHATE LEVEL

S1P levels are predominately controlled by three enzymes: sphingosine-1-phosphate phosphatase (S1P-Phosphatase), sphingosine kinase (SK), and sphingosine-1-phosphate lyase (S1P-lyase) (Oskouian & Saba, 2010) (Figure 7). S1P is synthesized through phosphorylation of Sph, and this reaction is catalyzed by SK. Then S1P undergoes irreversible cleavage by the action of S1P-lyase to ethanolamine phosphate and hexadecenal, or it can be reversibly dephosphorylated by S1P-phosphatase back to Sph.

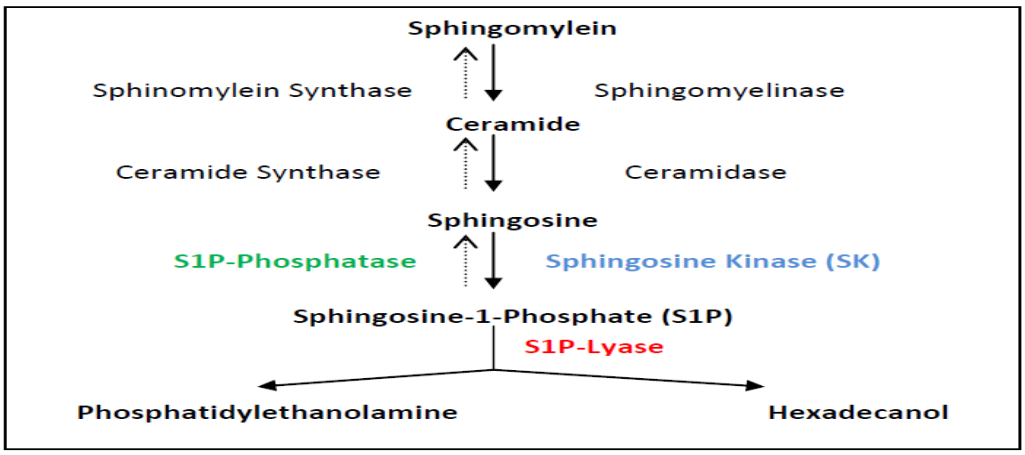


Figure 7. The enzymes that regulate sphingosine-1-phosphate level. S1P is synthesized through the phosphorolyation of sphingosine by sphingosine kinase (SK) enzyme and will undergo irreversible cleavage by the action of sphingosine-1-phosphate lyase (S1P-lyase) to ethanolamine phosphate and hexadecenal or it can dephosphorylated by sphingosine-1-phosphate phosphatase (S1P-phosphatase) back to sphingosine.

1.3.2.1. SPHINGOSINE KINASE

SK has two isoforms and both catalyze the phosphorylation of Sph by the same mechanism (Oskouian & Saba, 2010). SK1 is stimulated by hormones, growth factors, cytokines, chemokines, and immunoglobulin, whereas SK2 has fewer stimuli than SK1, and include EGF, IgE, IL-1β, and TNF-α (Oskouian & Saba, 2010). SK1 promotes cell survival and growth by

decreasing the level of Cer, which it does by inhibiting Cer synthesis and increasing the production of intracellular S1P that will be released to the extracellular surface by ABCC1 or ABCG2, where it can bind to the cell surface S1PRs (Oskouian & Saba, 2010; Takabe et al., 2010). Oddly, because it uniquely couples with S1P-phosphatase enzyme, SK2 counteracts the SK1 effect by inducing apoptosis through enhancing the Sph turnover in to Cer (Oskouian & Saba, 2010; Sabbadini, 2006). Studies have shown that SK1 and SK2 have different sub-cellular locations; SK1 is predominant in the cytosol and SK2 is present mainly in the nucleus and other sub-cellular compartments (Pyne & Pyne, 2010).

1.3.2.2. SPHINGOSINE-1-PHOSPHATE PHOSPHATASE

S1P can be dephosphorylated by the action of S1P specific phosphatases, known as S1P-phosphatase 1 and 2 (Gault, Obeid & Hannun, 2010). These phosphatases are present in the endoplasmic reticulum and they catalyze S1P recycling to Sph (Gault, Obeid & Hannun, 2010). S1P-phosphatases play a role in regulating the reintroduction of Cer at the endoplasmic reticulum and affects S1P signalling (Gault, Obeid & Hannun, 2010). Over-expression of the S1P-phosphatase enzyme is associated with promoting the accumulation of Cer and reduction in the extracellular S1P release (Gault, Obeid & Hannun, 2010). Therefore, these phosphatases determine the metabolic fate of S1P and they affect the biological responses of S1P in the cell (Gault, Obeid & Hannun, 2010).

1.3.2.3. SPHNGOSINE-1-PHOSPHATE LYASE

S1P-lyase enzyme causes irreversible conversion of S1P to hexadecenal and phosphoethanolamine and this represents the final step in the sphingolipids pathway (Gault, Obeid & Hannun, 2010). S1P-lyase enzyme is mainly present in the endoplasmic reticulum (Gault, Obeid & Hannun, 2010). S1P-lyase has broad substrate specificity; it degrades many

sphingolipid phosphate bases including S1P, diydrosphingosine-1-phosphate, and phytosphingosine-1-phosphate (Gault, Obeid & Hannun, 2010). S1P-lyase role needs more clarification. Some studies have shown that S1P-lyase plays a major role in tissue development and is associated with many disorders, such as immunological diseases and cancer (Gault, Obeid & Hannun, 2010).

1.3.3. SPHINGOSINE-1-PHOSPHATE DUAL EFFECT ON THE CELL

Numerous data have reported that S1P has dual effects on the cell. S1P is a unique sphingolipids and has a complex role compared to other sphingolipids. Many observations revealed that S1P has intrinsic and extrinsic effects on the cell (Figure 8). The intracellular actions are usually associated with its apoptotic effect, while S1P extracellular actions are correlated with its proliferation effect and survival. The dual effects are mainly due to two factors: the sub-cellular location of S1P formation and S1PRs subtypes expression in certain cell.

1.3.3.1. INTRACELLULAR ACTION

S1P's intracellular target is not well defined, but S1P acts as a second messenger regulating intracellular calcium mobilization (Hait et al., 2006). Studies suggest that S1P induces calcium release from the endoplasmic reticulum (Maceyke, Harikumar, Milstien & Spiegel, 2012). S1P produced in the nucleus by SK2 regulates histone acetylation and enhances their transcription (Maceyke, Harikumar, Milstien & Spiegel, 2012). In addition, the SK2 that is localized in the mitochondria produces mitochondrial S1P, which interacts with prohibitin 2 (PHB2) (Maceyke, Harikumar, Milstien & Spiegel, 2012). Therefore, mitochondrial S1P plays a role in mitochondrial respiration (Maceyke, Harikumar, Milstien & Spiegel, 2012). Although the intracellular effects of S1P are not well defined, many studies are correlating the apoptotic effect

of S1P to its intracellular action. More studies are needed to elucidate the intracellular role of S1P in the cell fate.

1.3.3.2. EXTRACELLULAR ACTION

S1P has the ability to activate cell surface $S1PR_{1-5}$ family of G-protein-couple receptors; these receptors are formally called endothelial differentiation gene (EDG) receptors (Oskouian & Saba, 2010). The S1PRs have different expression levels in different tissues depending on the cell type. This explains the diversity of S1P functions (Table 1). $S1PR_1$ and $S1PR_3$ activation promote cell survival, migration, and angiogenesis (Oskouian & Saba, 2010). In contrast, stimulation of $S1PR_2$ inhibits cell migration and increases vascular permeability (Oskouian & Saba, 2010). $S1PR_4$ and $S1PR_5$ have a narrow expression pattern compared to the other S1PRs, and their role and function is not clear (Oskouian & Saba, 2010).

In summary, the effects of S1P are determined mainly by the S1PRs sub-types predominantly expressed in certain tissues and in differences in the sub-cellular location of SK1 and SK2 (Pyne & Pyne, 2010). S1P is present in the plasma at a higher level (0.2-0.9 μM) than at the intracellular level, but it has high protein binding affinity to serum albumin and lipoprotein (Hannun & Obeid, 2008; Ling et al., 2011). The effect of the SK-S1P signalling pathway on the cell is complex and depends on many factors, such as cell type, receptor expression, and sub-cellular location of S1P formation (Pyne & Pyne, 2010).

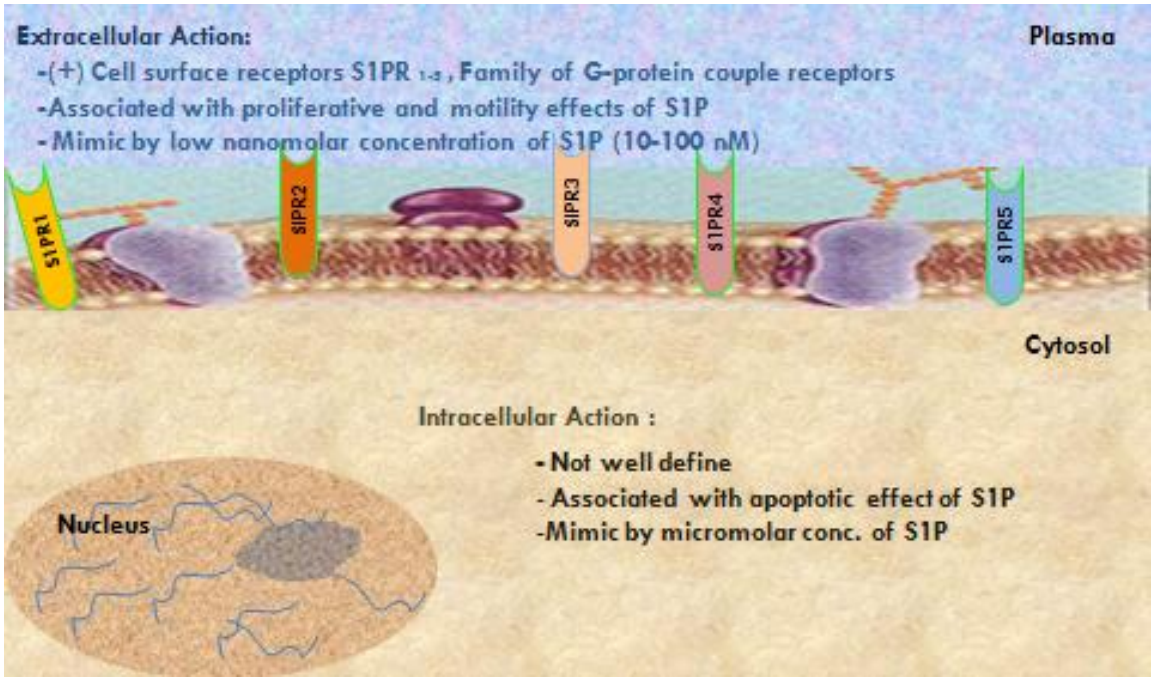


Figure 8. The dual effect of sphingosine-1-phosphate (S1P) on cell fate. S1P exerts its proliferation effect through the stimulation of cell surface receptors (i.e. S1PRs) and its apoptosis effect through its intracellular action by unknown mechanism.

Table1. Summary of sphingosine-1-phosphate receptors (S1PRs) tissue distribution and function:

S1PRs subtype	Tissue Distribution	Function
$SIPR_1$	Brain Lung Spleen Liver Heart Kidney Skeletal muscle Thymus Lymphoid	1- Angiogenesis, vascular integrity 2- Stimulate immune cell function, control lymphocyte recirculation 3- Increase cell migration 4- Enhance cell proliferation and survivor
$S1PR_2$	Brain Lung Spleen Liver Heart Kidney Skeletal muscle Thymus	1- Inhibit cell migration 2- Increase vascular permeability 3- Promotes liver tissue remodelling in response to acute injury
$S1PR_3$	Brain Lung Spleen Liver Heart Kidney Skeletal muscle Thymus Testis	1- Increase cell migration 2- Vascular integrity 3- Regulate the Heart Rate 4- Enhance cell proliferation and survivor
$S1PR_4$	Lymphoid tissue Leukocyte Bone Marrow Spleen Thymus	unclear
$S1PR_5$	Oligodendrocyte Natural Killer Cells Brain Spleen Skin	unclear

1.3.4. BREAST CANCER AND SPHINGOSINE-1-PHOSPHATE:

A number of *in vitro* studies have been done to study the effect of S1P on different breast cancer cell lines and the results are remarkable. These studies found that S1P enhances the cytotoxicity of anticancer drugs (Ling et al., 2011), and inhibits motility and proliferation of breast cancer cells (Wang et al., 1999).

The study conducted by Ling et al. found that exogenous administration of S1P can improve the docetaxel treatment potency in MCF7 breast cancer cells. The IC_{50} value of docetaxel alone was 3.4 µg/ml and its value when co-administrated with 1 µM S1P was 2.1 µg/ml. This means that S1P enhances the cytotoxicity of docetaxel against MCF7 breast cancer cells. In addition, in the same study Ling et al. found that S1P shows different apoptotic responses in MCF7 (breast cancer cells line) compared to MCF12A (normal breast cells line), and this suggests that its effect could be selective to cancer cells (Ling et al., 2011).

Another study done by Wang et al. examined the involvement of S1PRs in the motility inhibition following exogenous administration of S1P on the following breast cancer cell line: MCF7, ZR75-1, MDA-MB 231, and BT 549. These researchers found that the S1P inhibition of the motility was independent of the receptor and was associated with the intracellular action of S1P. Following the administration of S1P, they measured its intracellular levels in the sample and found it much higher than the control; this suggests that S1P will inhibit cell motility by intracellular actions independent of S1PRs (Wang et al., 1999).

Spiegel et al. found similar results to Wang et al.'s study regarding S1P's effect on the breast cancer cells' motility. Their study showed that S1P at (1- 10 µM) concentration can inhibit the motility and proliferation of human breast cancer cell lines (MCF7 and MDA-MB 231), and that

S1P is more potent against more aggressive estrogen-independent breast cancer cells, MDA-MB 231, compared to its effect on estrogen-dependent breast cancer cells, MCF7, which are less aggressive. This means that S1P could be a useful agent in suppressing the breast cancer metastasis in more aggressive breast cancer cells (Spiegel et al., 1994).

From all of these studies, it is evident that S1P could be a good candidate for breast cancer treatment since it inhibits the cells metastasis and proliferation, induces apoptosis, and enhances the cytotoxicity of anti-cancer drugs like docetaxel.

1.3.5. SPHINGOSINE-1-PHOSPHATE APOPTOTIC EFFECT:

Because many studies associate S1P with cell proliferation and survival, a number of drugs have been developed to inhibit S1P synthesis by blocking the SK enzyme. Other studies have found that S1P plays a major role in inducing apoptosis, showing that S1P has a dual effect on cell fate (Oskouian & Saba, 2010). This new finding has led to the possibility of future evaluations of the SK-S1P pathway and the conclusion that S1P generated from SK1 is associated with cell proliferation and needs activation of S1PRs, while S1P generated from SK2 is correlated with the intracellular apoptotic effect (Oskouian & Saba, 2010).

Davaille et al. examined the S1P proliferation effect on human hepatic myofibroblasts (hMF) and found that S1P exhibits potent inhibition of hMF proliferation: the IC_{50}= 1 µM. In this study, they linked the anti-proliferation effect of S1P to its intracellular action and suggested the involvement of COX2. They found that S1P will induce COX2 expression as part of its mechanism of growth inhibition. They blocked the COX2 by dexamethasone and as a result the anti-proliferation effect of S1P was abolished. Also, this research group further found that S1P has a dual opposing effect on hMF. S1P can enhance the cell proliferation of hMF by activating

S1PRs at a nanomolar concentration and the apoptotic effect occurred at a micromolar concentration of S1P. In the same study, they found that S1P apoptotic effect is not related to its conversion to Sph or Cer. They examined the effects of Sph and Cer and found that S1P exerts its apoptotic effect *via* a caspase-dependent mechanism and that Sph stimulates apoptosis in a caspase-independent mechanism (Davaille et al., 2000; Davaille et al., 2002).

Hung and Chuang found that S1P can induce apoptosis in human hepatoma cells (Hep3B) by increasing the expression of the Bax gene (Hung & Chuang, 1996). Moore et al. found that prolonged exposure to 10 μM S1P induces apoptosis in hippocampal neurons by increasing the level of intracellular Ca^{2+}. S1P will enhance the release of Ca^{2+} from IP_3-senestive stores. Also, they found that prolonged exposure to low levels of S1P (2 μM) did not cause any morphological change (Moore et al., 1999).

All of these studies support the hypothesis that the S1P apoptotic effect is correlated with its intracellular action. Until now studies did not identify the S1P intracellular target that mediates its intracellular effects.

1.4. HYPOTHESIS

We are proposing sphingosine-1-phosphate (S1P) as a target for breast cancer treatment and prevention:

I. S1P induces apoptosis and necrosis in breast cancer cells and inhibits breast cancer cells proliferation.

II. S1P enhances the cytotoxicity of anti-cancer drugs against human breast cancer cells *in vitro* and *in vivo*.

III. S1P apoptosis effect is correlated with it intracellular action.

1.5. OBJECTIVES

I. Examine whether exogenous administration of S1P will selectivity induce apoptosis, necrosis and inhibit proliferation in breast cancer cell lines.

II. Evaluate the therapeutic effect of S1P alone or in combination with anti-cancer drugs against breast cancer by using MCF7 xenograft mouse model (*in vivo*) and MDA-MB 231 and MDA-MB 361 breast cancer cell line (*in vitro*).

III. Study the cell distribution of S1P following exogenous administration by using Raman imaging

2. MATERIALS AND METHODS:

2.1. MATERIALS:

Sphingosine-1-phospahte (S9666- 1 mg), docetaxel (01885- 5 mg), doxorubicin (D1515- 10 mg) and cyclophosphamide (C0768- 1 gm) were purchased from Sigma-Aldrich (Oakville, ON, Canada). All the cells lines were purchased from American Type Culture Collection (ATCC) (Manassas, VA, USA): MDA-MB 231 cells (source: human breast adenocarcinoma, drive from pleural effusion), MDA-MB 361 cells (source: human breast adenocarcinoma, drive from metastatic brain), MCF7 cells (source: human breast adenocarcinoma, drive from pleural effusion) and the MCF12A cells (source: human mammary gland; breast). Penicillin/streptomycin solution was purchased from Sigma Life Science (Kansas city, MO, USA). Leibovitz's L-15 media, Eagle's Minimum Essential media, and 1:1 mixture of Dulbecco's modified Eagle's medium with ham's F12 media and Fetal Bovine Serum (FBS) were purchased from ATCC. 0.25 % Trypsin EDTA, bovine insulin, human epidermal growth factor, cholera toxin, and hydrocortisone were purchased from Sigma Life Science. Horse serum was from ATCC. CellTiter 96® AQ_{ueous} Non-Radioactive Cell Proliferation Assay, Caspase-Glo® 3/7 Assay System, and CytoTox 96 ® Non-Radioactive Cytotoxicity Assay System were from Promega (Madison, WI, USA). Innovation Research of American (Sarasota, FL, USA) provided 0.72 mg 17-β Estradiol pellet with 60-day release. The Matrigel matrix came from BD Bioscience (Franklin lakes, NJ, USA). Balb/c female nude mice were obtained from Charles River Laboratories Canada, Inc (Senneville, QC, Canada).

2.2. COMPOUNDS AND BUFFERS PREPARATION:

Stock solutions were prepared for S1P dissolved in methanol at a 2.64 mM; docetaxel in ethanol at a 2 mg/mL; doxorubicin in water at a 1 mM; and cyclophosphamide in ethanol at a

100 mM. Additionally, 1x PBS buffer (pH 7.4) was prepared by dissolving the following chemicals in 800 mL distilled water: 8 g NaCl, 0.2 g KCl, 1.44 g of Na_2HPO_4, and 0.24 g of KH_2PO_4. The pH was adjusted to 7.4 by adding HCL and then volume was adjusted to 1L. The final PBS solution was sterilized by autoclaving. 4% paraformaldehyde was prepared by dissolving 4gm of paraformaldehyde powder in 100 mL of 1x PBS and the final solution was heated at 60-70℃ water bath for one-two hours.

2.3. CELL CULTURE:

The cells were allowed to grow in monolayer (75 cm^2 or 150 cm^2 tissue culture flasks) and were cultured according to ATCC instructions (Table 2). The complete growth media for each cell line was supplemented with 1% penicillin/streptomycin solution. For MDA-MB 231 cells, I used Leibovitz's L-15 media and supplemented it with 10% FBS; for MDA-MB 361 cells, I supplemented Leibovitz's L-15 media with 20% FBS; for MCF7 cells and added 10% FBS and 0.01 mg/ml bovine insulin to Eagle's Minimum Essential medium; and for MCF12A cells, I used 1:1 mixture of Dulbecco's modified Eagle's medium and ham's F12 medium and I supplemented it with 20 ng/ml human epidermal growth factor, 100 ng/ml cholera toxin, 0.01 mg/ml bovine insulin, 500 ng/ml hydrocortisone, and 95% horse serum. The MDA-MB 231 and MDA-MB 361 cells were incubated in a humidified cell culture incubator at 37˚C, without CO_2, while the MCF7 and MCF12A cells were incubated in the same condition but with 5% CO_2. The media for all cultured cells was changed every two to three days. The cells were sub-cultured when they reached more than 80% confluency by using 0.25% trypsin EDTA 1X solution.

2.4. CELL PROLIFERATION ASSAY:

96-well tissue culture plates were seeded with MDA-MB 231 cells at a density of 7000 cells/ well, while the MDA-MB 361 cells were seeded at 10,000 cells/well. When the cells reached 60-

70% confluency, they were treated with S1P (concentration range: 0.125- 8 µM) or methanol, and then incubated in 37˚C, without CO_2 for 18- 19 hours. After that, I performed the assay by using the CellTiter 96® AQ_{ueous} Non-Radioactive Cell Proliferation Assay kit, following the manufacturer's protocol. In the final step, I used a BioteK plate reader to measure the absorbance at 490 nm. This assay is a colorimetric method used to determine the number of living cells by measuring the quantity of Formazen. The Formazen is formed as a result of the conversion of MTS (i.e. 3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium)), which is found in the assay solution. This conversion is catalysed by the dehydrogenase enzyme, which is usually found in metabolically active cells. The data were plotted as % increase of cell proliferation compared to the control vs. S1P concentration and data was analyzed using GraphPad Prism software. For the time response assay, I did the same previous step but treated cells with 1, 10 µM S1P or methanol one time. Proliferation was measured at different time points (at 0, 6, 12, 24, 48, and 72 hours) and the final data were plotted as % increase of cell proliferation vs. time in hours.

2.5. APOPTOSIS ASSAY:

A total of 7000 MDA-MB 231 cells/well, 10,000 MDA-MB 361 cells/well, or 5000 of MCF12A cells/well were seeded in a white 96-well tissue culture plate. When the cells reached 60- 70% confluency, they were treated with S1P (concentration range: 0.15625- 40 µM) or methanol; the treated cells were incubated at 37˚C, humidified, without CO_2 for 48 hours for MDA-MB 231 cells and 18 hours for MDA-MB 361 cells, while the MCF12A was treated for 24 hours at 37˚C, humidified, in 5% CO_2. After that, I performed the apoptosis assay by using Caspase-Glo® 3/7 Assay System kit, following the manufacturer's protocol. Later I used a BioteK plate reader to measure the luminescence that represented the caspase3/7 activity. This

assay is a homogenous luminescent assay, which measures the caspase 3/7 activity. The assay solution contains proluminescent caspase-3/7 DEVD- aminoluciferin substrate and proprietary thermostable luciferase. The presence of caspase 3/7 in the cell culture will cleave the substrate and free the aminoluciferin. The free aminoluciferin will be consumed by the luciferase enzyme to give a glow luminescence. For the calculation, I used the cells treated with solvent (i.e., methanol) without S1P as a control. Data were plotted as % increase of cells apoptosis compared to control vs. S1P concentration and I used GraphPad Prism software for my data analysis; to calculate the IC_{50} values I used the nonlinear regression analysis, log [agonist] vs. response equation.

2.6. CYTOTOXICITY STUDIES:

For this study, 96-well plates were seeded with 7000 MDA-MB 231 cells/well, 10,000 MDA-MB 361 cells/well, or 5000 MCF12A cells/well. When the cells reached 60- 70% confluency, they were treated with S1P at concentration range of 0.15625- 40 µM to determine the cytotoxicity effect of S1P on these cell lines. I performed another cytotoxicity study on MDA-MB 231 and MDA-MB 361 cells. Here, I treated the cells with a different concentration of the anti-cancer drugs alone or in combination with 1 or 10 µM S1P as follow: docetaxel (concentration: 40- 0.625 µg/ml), doxorubicin (concentration: 20- 0.3125 µM), or cyclophosphamide (concentration: 2- 0.0325 mM). After that, I incubated the treated cells for 18-19 hours for MDA-MB 231 and MDA-MB 361 cells, and 24 hours for MCF12A. In the next step, I performed the cytotoxicity assay by using CytoTox 96 ® Non-Radioactive Cytotoxicity Assay kit. In the end, I used a Biotek plate reader and I quantitatively measured lactate dehydrogenase (LDH), which is a cytosolic enzyme released upon cell lysis, by recording the

absorbance at 490 nm. The background absorbance was corrected by including a negative control and I determined the % cell death by using the following formula:

$$\% \text{ Cytotoxicity} = \frac{Expermint - Control}{Maximum\ LDH\ release\ (i.e. positive\ control) - Control} \times 100$$

The data were plotted as % cytotoxicity vs. S1P concentration for the first assay and vs. each anti-cancer drug concentration for the second assay; I used GraphPad Prism software to evaluate the data and I calculated the IC_{50} values by using the nonlinear regression analysis, log [agonist] vs response equation. To compare between the effects of the anti-cancer drugs alone or in combination with S1P I performed a t-test. For the time response assay, I treated the MDA-MB 231 and MDA-MB 361 with 1, 10 µM S1P or methanol, one time treatment and I used the same previous steps and % cytotoxicity was measured at different time points (at 0, 6, 12, 24, 48, and 72 hours).

2.7. RAMAN IMAGING:

The MCF7 cells were seeded at a density of 50,000 cells/well in a 45-well plates that contained a gold plate. I allowed the cells to grow on the gold plate for 24 hours, and then I treated the cells with 10 µM S1P for 12 hours. After that, I removed the media and washed the gold plate twice with1xPBS for both the control and the treated sample. Then I placed the gold plate in a Petri dish containing 2 ml 1xPBS, and I used the Raman spectrophotometer to detect the spectrum of the control and treated cells. When I found the S1P signal I did the mapping at the higher peak of S1P intensity signal, i.e. 1300 Raman shift/ cm^{-1}, see Figure 20. Also, I measured the pure S1P powder extended and static spectrum and compared it with the experiment spectrum; although the sample signal is weak, it fit with the pure S1P signal.

2.8. ANIMAL STUDIES:

This work was approved by the University of Saskatchewan's Animal Research Ethics Board and adhered to the Canadian Council on Animal Care guidelines for humane animal use. Thirty 4- 6 week old Balb/c female nude mice were housed in ventilated cages under sterile conditions, and sterile food and water were provided to them *ad libitum*. I used MCF7 cell line passage no. 151 to develop the xenograft nude mice model. When the cells reached 70- 80% confluency, I injected 2- 5 x 10^6 of MCF7 cells suspended in 1:1 of (1xPBS and Matrigel matrix) subcutaneously in the mammary fat pad of the nude mice by using a 1 cc syringe and a 27-guage needle. We implanted 0.72 mg of the 17-β Estradiol 60-day release pellet beneath the lateral side of each mouse's neck between the ear and the shoulder one day before the injection of the cells and this surgery was done while the mice are under isoflurane anesthesia. We implant the estrogen because MCF7 cells are estrogen-dependent breast cancer cells and estrogen facilitates its growth. The mice were monitored every three days during the tumour induction period. I started the treatment after 38 days post-cell injection. The tumour length and width was measured by using a digital calliper and the following formula was applied to calculate the tumour volume: $V= \frac{\pi}{6}$ (width x length) $^{3/2}$. Only 14 mice developed the tumour which means that the tumour induction success rate is consistent with the literature which is usually (40- 60%). I assigned the 14 mice in to four different groups as follows: the first group was the control group (n= 2) treated with the 1xPBS only; the second group (n= 4) was treated with 1 µM S1P; the third group (n=4) was treated with 6 µg Docetaxel; and the fourth group (n=4) was treated with a combination of 1 µM S1P and 6 µg Docetaxel. I grouped the nude mice according to their weight and the weight difference in each group is less than 1 gram. All agents were given as intra tumoural injections with a total volume of 100 µL by using 27-gauge needles. Injections were

given every other day for 21 days (i.e., 10 doses were given). Tumour diameters, weight, and the life-span of the control and treated mice were recorded daily during the experiment.

2.9. IMMUNOHISTOCHEMISTRY STUDIES:

At the end of the animal experiment, all remaining mice were sacrificed and hearts, livers, kidneys and tumours were harvested. The collected tissues were embedded at 10x their volume in 4% paraformaldehyde for fixation and stored in the refrigerator for 72 hours. All tissues were washed with 70% ethanol before processing. The following day, I blocked the tissue in wax and I started sectioning the wax blocks in to 5 mm slices. Then I stained the slices with haematoxylin and eosin.

2.10. STATISTICAL ANALYSIS:

I used GraphPad Prism software for my data analysis. The mean ± SD for each assay was calculated from three independent experiments. To calculate the IC_{50} values I used the nonlinear regression analysis, log [agonist] vs. response equation. To compare between the effects of the anti-cancer drugs alone or in combination with S1P I performed a t-test. In the animal study I performed a One-Way ANOVA followed by Tukey's and Bonferroni's multiple comparison tests to compare between the four groups.

Table 2. Cell culture information summary:

Cell line	Ethnic	Gender	Age (years Old)	Gene Cluster	Tumour Type	Receptor Expression				Source	Passage no.	Culture Media	Incubation conditions
						ER	PR	HER2	TP53				
MDA-MB 231	Cauc.	♀	51	Basal	AC	_	_	_	+	Pleural effusion	31	L-15, 10% FBS	37˚C, No CO_2
MDA-MB 361	Cauc.	♀	40	Luminal	AC	+	_	+	_	Brain	33	L-15, 20 % FBS	37˚C, No CO_2
MCF7	Cauc.	♀	69	Luminal	AC	+	+	_	+/_	Pleural effusion	149	MEM, 10% FBS	37˚C, 5%CO_2
MCF12A	Cauc.	♀	60	Basal	F	_	_		+	Pleural effusion	9	DMEM/F12*	37˚C, 5%CO_2

AC: Adenocarcinoma, Cauc: Caucasian, ER: Estrogen Receptors, PR: Progesterone Receptors, HER2: Human Epidermal Growth Factor Receptor 2, TP53: Tumour Protein 53, L-15: Leibovitz's L-15 medium, MEM: Eagle's Minimum Essential medium, DMEM/F12: Dulbecco's modified Eagle's medium and Ham's F12 medium, FBS: Fetal Bovine Serum.

3. RESULTS

3.1. CELL PROLIFERATION ASSAY:

As shown in Figure 9, S1P produced a bell-shaped dose response curve on human breast cancer cell lines MDA-MB 361 and MDA-MB 231. Maximal proliferation was achieved at 1 µM and 2 µM in MDA-MB 361 and MDA-MB 231 cells, respectively. S1P caused a stimulatory response at a concentration lower than 2 µM and an inhibitory response at a higher concentration in MDA-MB 231 cells. In MDA-MB 361 cells, S1P caused a dramatic inhibition of cell proliferation at a concentration higher than 4 µM. In the time response study (Figure 10) prolonged exposure to high concentrations of S1P (i.e. 10 µM) significantly inhibited cell proliferation at a higher rate compared to lower concentrations (i.e. 1 µM) in both cell lines. Also, we observed that S1P was more effective against aggressive breast cancer cells (i.e. MDA-MB 231 cells, ER-ve/ HER-2 -ve) compared to less aggressive cell line (i.e. MDA-MB 361 cells, ER +ve/ HER-2 +ve) as shown in Figure 10. Exposure of these breast cancer cells with 10 µM S1P for 72 hours inhibited the proliferation in both cell lines. However, in the MDA-MB 231 cells the percent inhibition of proliferation was equal to 21%, while in MDA-MB 361 cells the percent inhibition was equal to 4%.

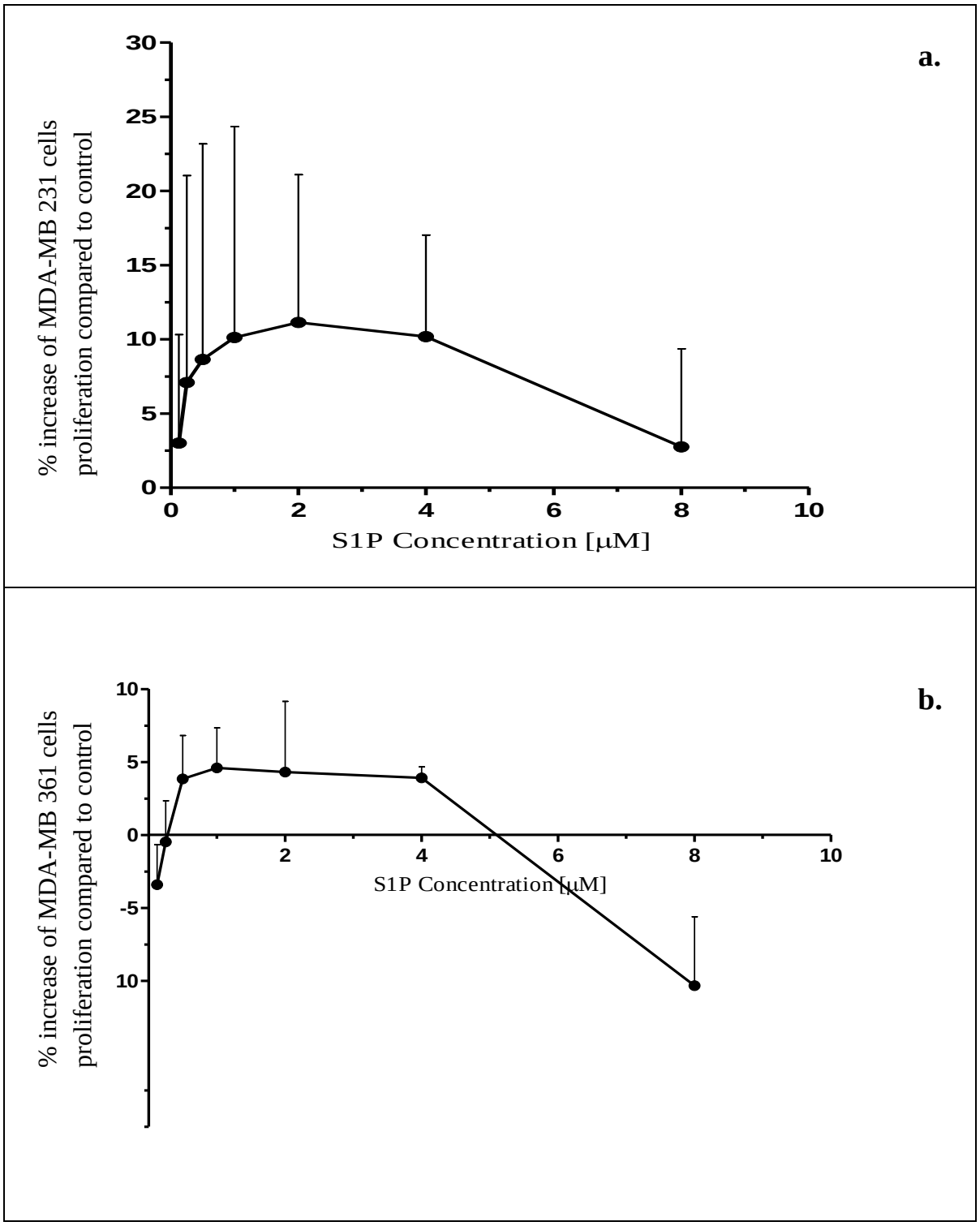


Figure 9. Percent increase of cellular proliferation (mean ± SD) compared to control of human breast cancer MDA-MB 231 cells (panel **a**) and MDA-MB 361 cells (panel **b**) following 18-19 hr treatment with different concentrations of S1P. Cells treated with the complete growth media and the solvent was used as a control. The mean ± SD was calculated from three independent experiments.

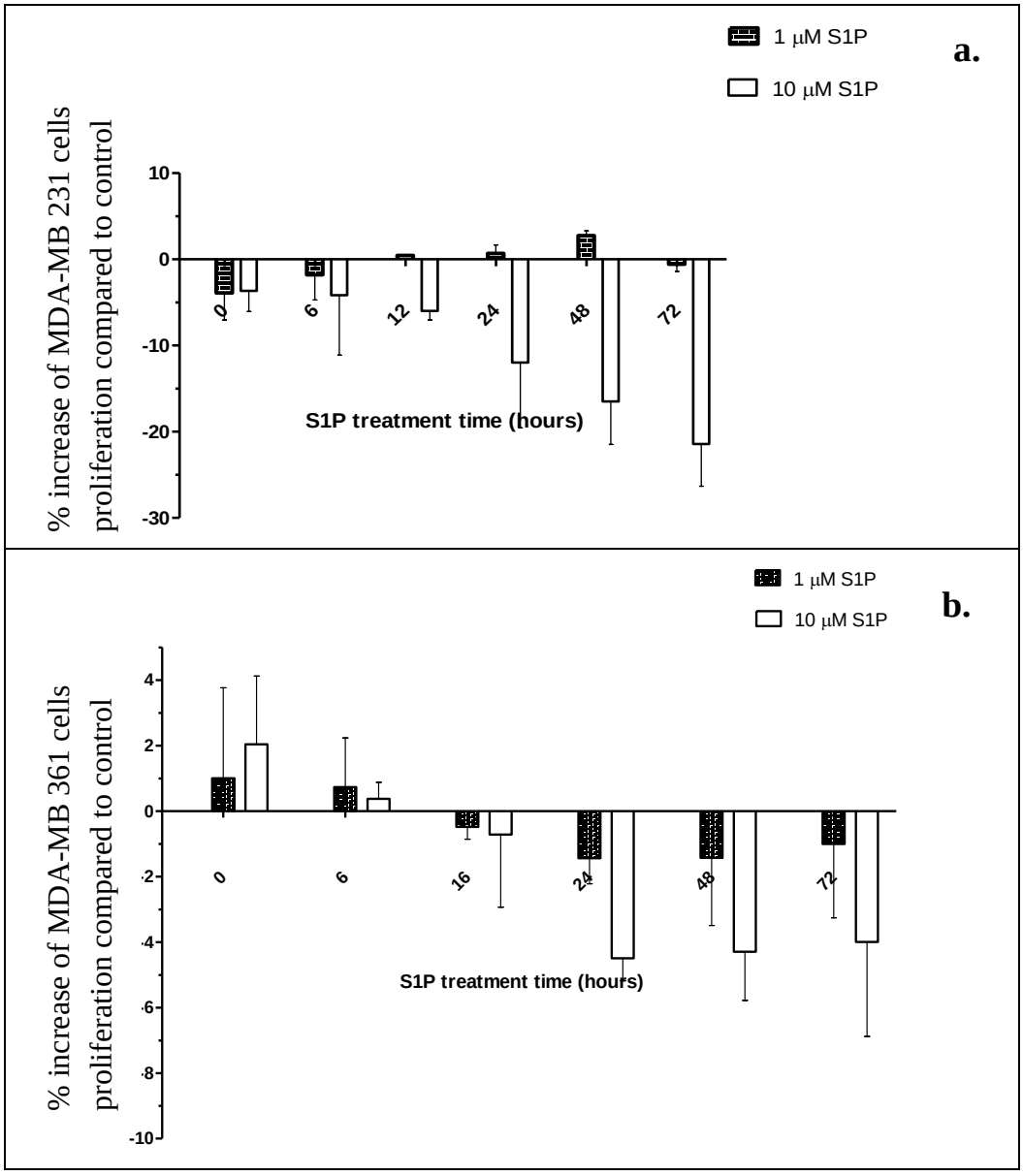
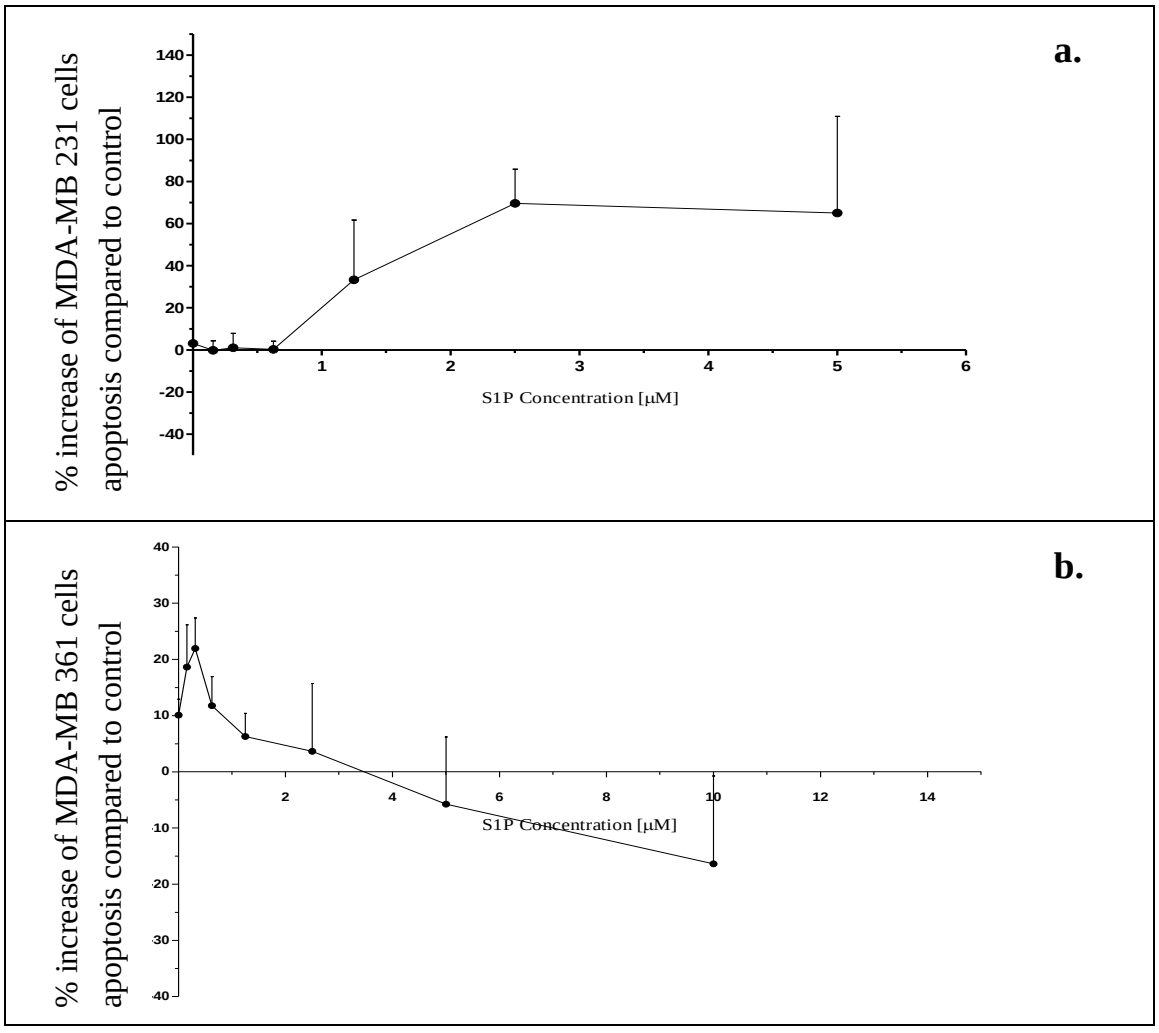


Figure 10. Percent increase of cellular proliferation (mean ± SD) compared to control of human breast cancer MDA-MB 231 cells (panel **a**) and MDA-MB 361 cells (panel **b**). The cells were treated with 1, 10 μM S1P or methanol and cellular proliferation was measured at different time points (at 0, 6, 12, 24, 48, and 72 hr). Cells treated with the complete growth media and the solvent was used as a control. The mean ± SD was calculated from three independent experiments.

3.2. CELL APOPTOSIS ASSAY:

The S1P IC_{50} ±SD in MDA-MB 231 breast cancer cells was 1.5 ±0.3 μM and in normal mammary epithelial MCF12A cells was 10.1± 5.0 μM as shown in Table 3 and Figure 11. This suggests greater apoptosis to cancer cells lines. As well, figure 11 (panel b) shows that S1P produced a bell-shaped dose response curve in MDA-MB 361 cells, with the maximal cell apoptosis at 0.3 μM.



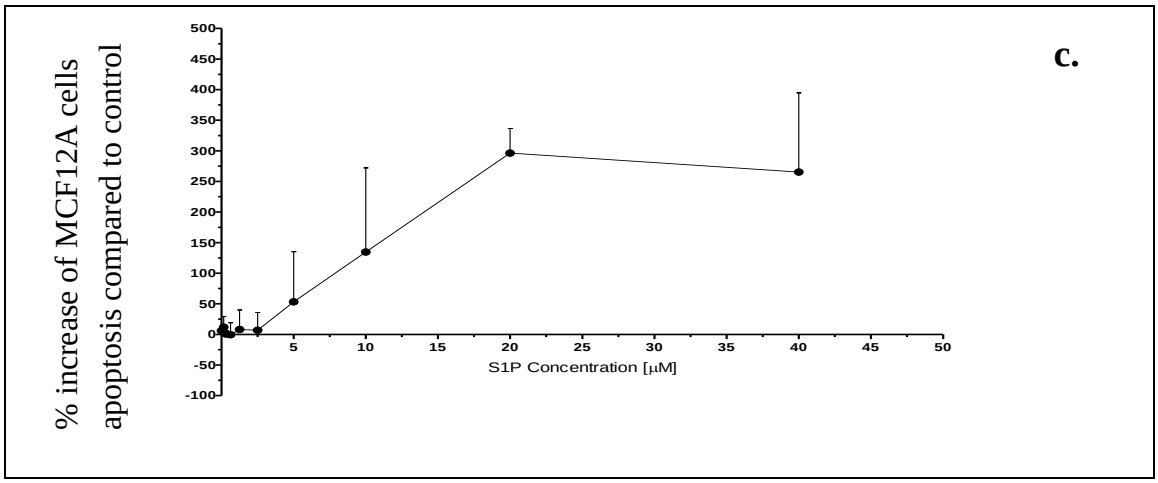


Figure 11. Percent increase of apoptosis (mean ± SD) compared to control of human breast cancer cells , MDA-MB 231 cells (panel **a**) and MDA-MB 361 cells (panel **b**), and normal breast cells MCF12A (panel **c**), following treatment with different concentration of S1P for 48 hr (**a**), 18 hr (**b**) or 24 hr (**c**). Cells treated with complete growth media and the solvent were used as a control. The IC_{50}±SD was calculated from three independent experiments, see Table 3.

Table 3. Summary of the sphingosine-1-phosphate half maximum inhibitory concentration results from the apoptosis and cytotoxicity studies:

Cell Line	IC_{50} ± SD *	
	Apoptosis	Cytotoxicity
MDA-MB 231 cells	1.5 ± 0.3	8.5 ± 4.1
MDA-MB 361 cells	_	14 ± 1.2
MCF 12 A	10.1 ± 5	13.2 ± 5.6

*IC_{50} shown for S1P is in μM. The cytotoxicity was determined by using Cytotox kit from Promega and apoptosis was determined by Caspase 3/7 kit as described in materials and methods section. The IC_{50} was calculated by using GraphPad Prism software and mean ± SD of three independent experiments was used.

3.3. CYTOTOXICITY STUDIES:

S1P caused cytotoxicity in MDA-MB 231 cells at a lower concentration compared to MDA-MB 361 and MCF12A cells, suggesting that S1P is more effective against MDA-MB 231 cells compared to MDA-MB 361 cells and that S1P can selectively kill MDA-MB 231 cells without harming normal breast cells as shown in Figure 12 and Table 3. In the time response study (Figure 13), prolonged exposure to higher concentrations of S1P caused higher cytotoxicity compared with lower concentrations and the effect was time dependent. S1P at 1 μM did not cause necrosis in normal and breast cancer cells. In Figures 14, 15, 16, and 17 S1P significantly enhanced the cytotoxicity of anti-cancer drugs docetaxel, doxorubicin and cyclophosphamide. In MDA-MB 231 cells, 1 and 10 μM of S1P enhanced the activity of docetaxel, doxorubicin, and cyclophosphamide significantly the p-value <0.05. In MDA-MB 361 cells S1P enhanced the cytotoxicity of chemotherapies only at 10 μM, which is consistent with previous results where S1P was shown to be more effective against MDA-MB 231 cells compared with MDA-MB 361 cells. In Table 4 summarizes the IC_{50}±SD values of anticancer drugs alone and in combination with S1P at an apoptosis dose (i.e., 1 μM) and necrosis dose (i.e., 10 μM). The data demonstrates the IC_{50} of anticancer drugs decreased significantly when co-administrated with 10 μM S1P compared with 1 μM S1P in both cell lines as shown in Figure 18 and 19.

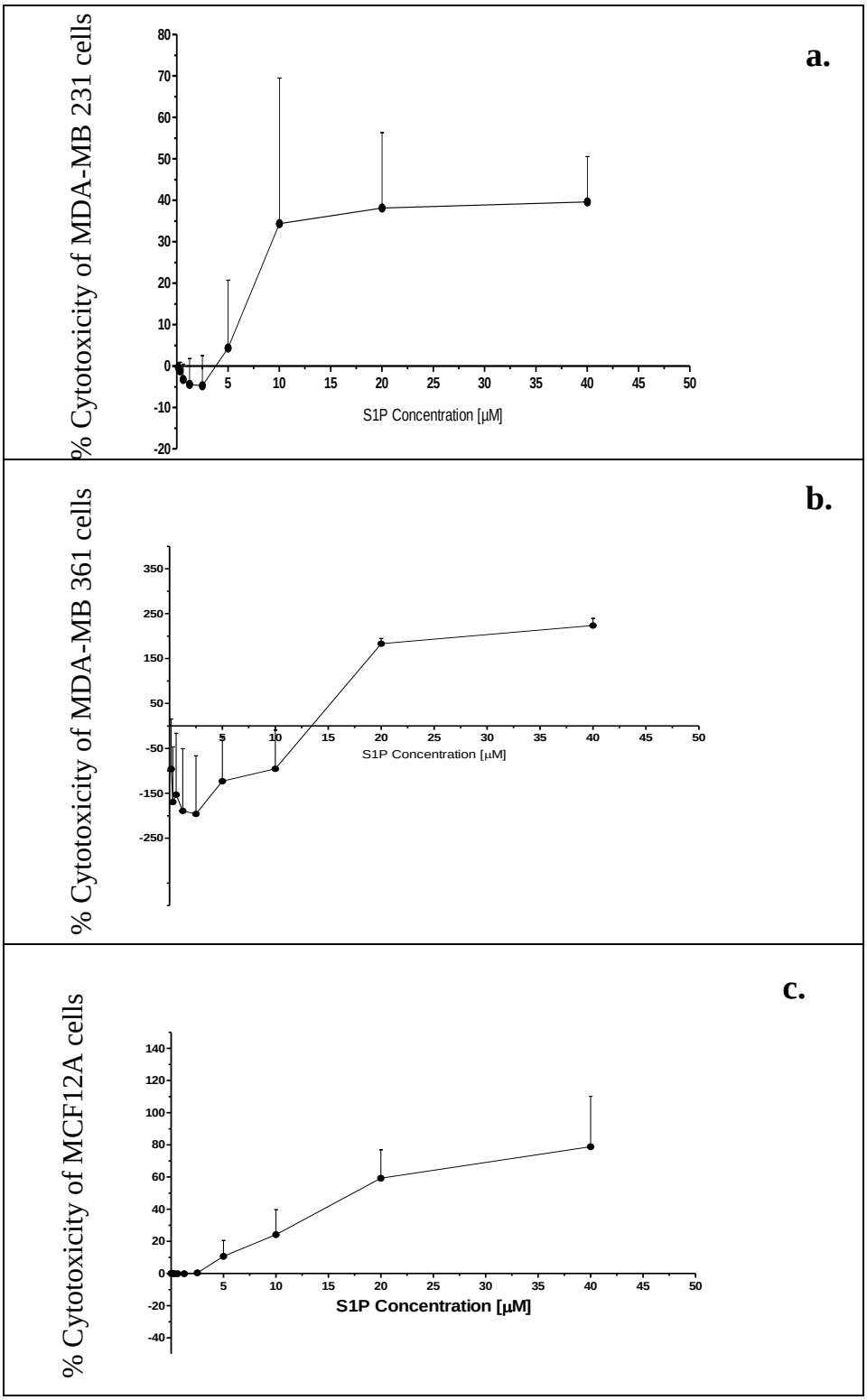


Figure 12. Percent cytotoxicity (mean ± SD) compared to control of human breast cancer cells , MDA-MB 231 cells (panel **a**) and MDA-MB 361 cells (panel **b**), and normal breast cells MCF12A (panel **c**), following treatment with different concentrations of S1P for 48 hr (**a**), 18 hr (**b**) or 24 hr (**c**), respectively. Cells treated with complete growth media and the solvent were used as a control. The IC_{50}±SD was calculated from three independent experiments, see Table 3.

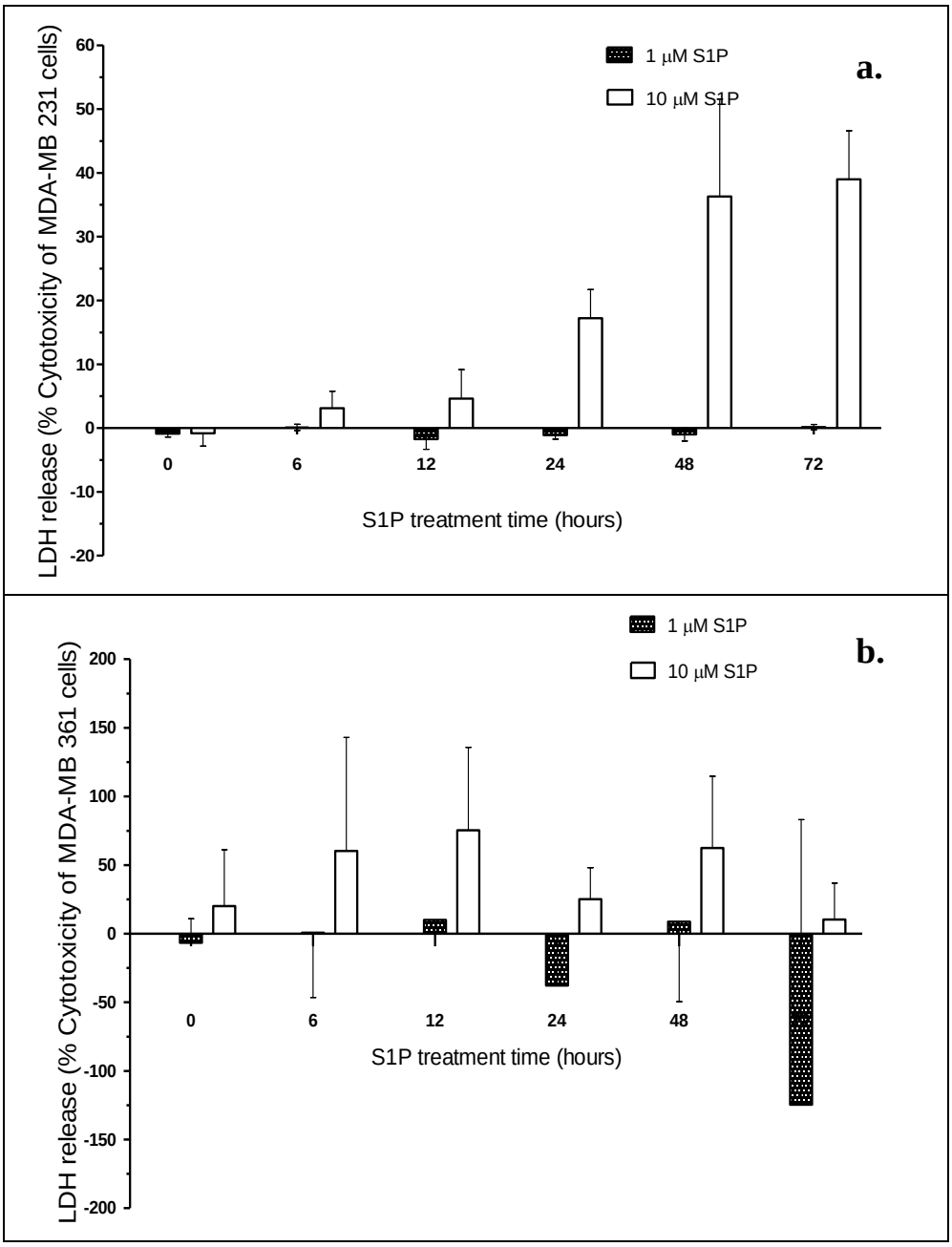


Figure 13. Percent cytotoxicity (mean ± SD) compared to control of human breast cancer MDA-MB 231 cells (panel **a**) and MDA-MB 361 cells (panel **b**). The cells were treated with 1, 10 μM S1P or methanol and the LDH release was measured at different time points (at 0, 6, 12, 24, 48, and 72 hr). Cells treated with the complete growth media and the solvent was used as a control. The mean ± SD was calculated from three independent experiments.

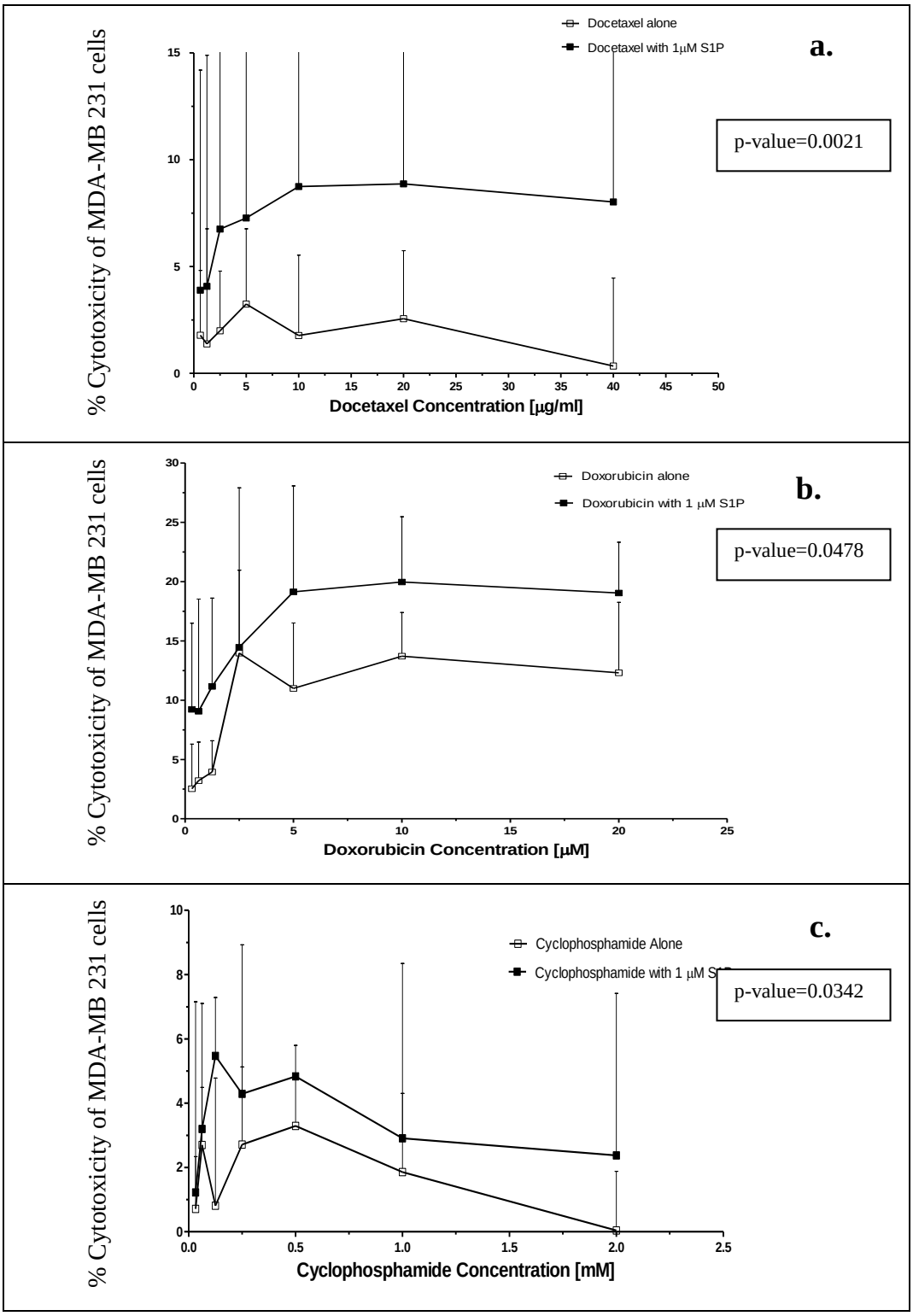


Figure 14. Percent cytotoxicity (mean ± SD) of the anticancer drugs against human breast cancer MDA-MB 231 cells alone (▭) or in combination with 1 μM S1P (■), docetaxel (panel **a**), doxorubicin (panel **b**), and cyclophosphamide (panel **c)**. The cytotoxicity was measured 18 hours after the treatment. Cells treated with the complete growth media and the solvent were used as the control. The mean ± SD was calculated from three independent experiments.

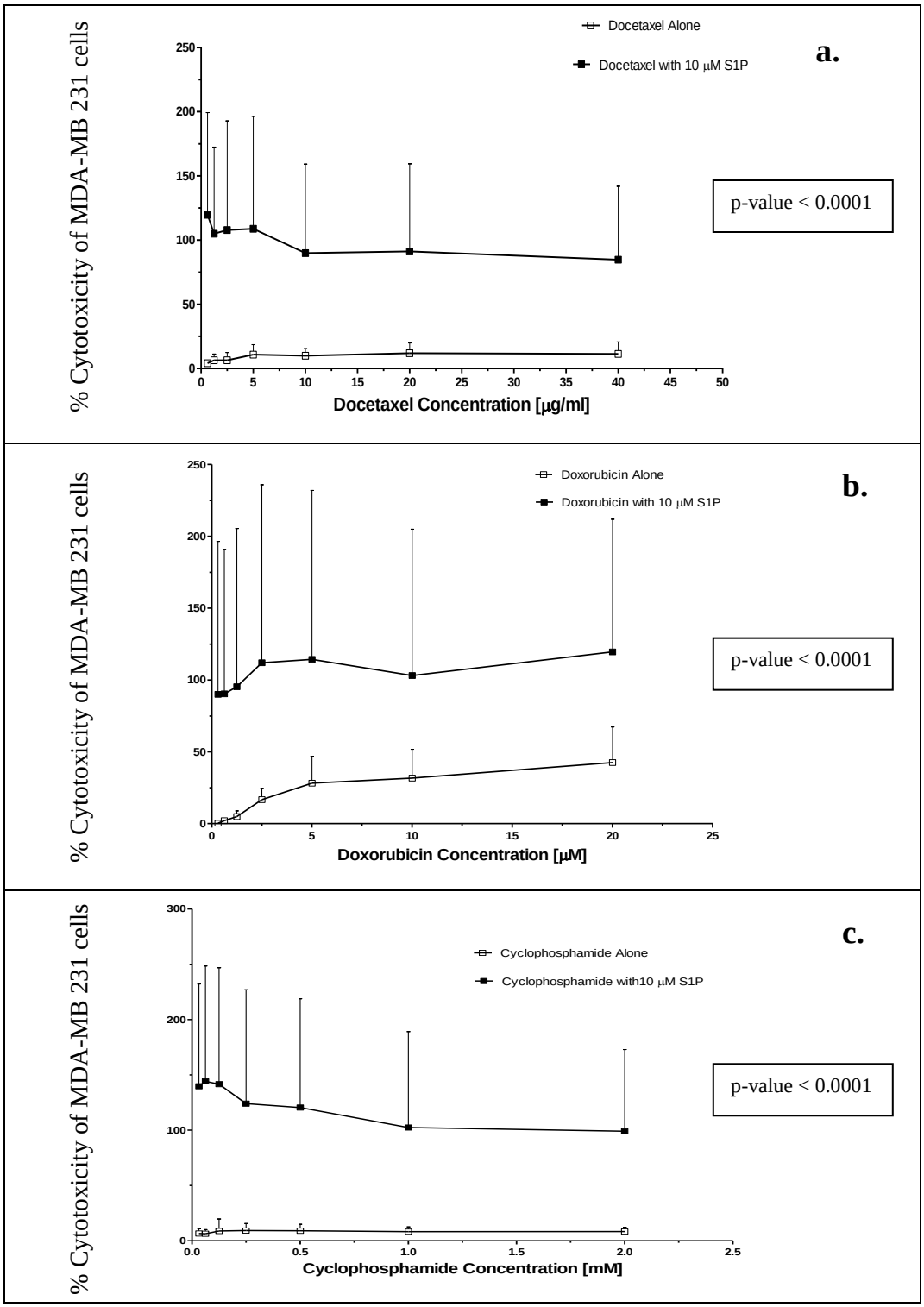


Figure 15. Percent cytotoxicity (mean ± SD) of the anticancer drugs against human breast cancer MDA-MB 231 cells alone (▭) or in combination with 10 μM S1P (▬), docetaxel (panel **a**), doxorubicin (panel **b**), and cyclophosphamide (panel **c)**. The cytotoxicity was measured 18 hours after the treatment. Cells treated with the complete growth media and the solvent were used as the control. The mean ± SD was calculated from three independent experiments.

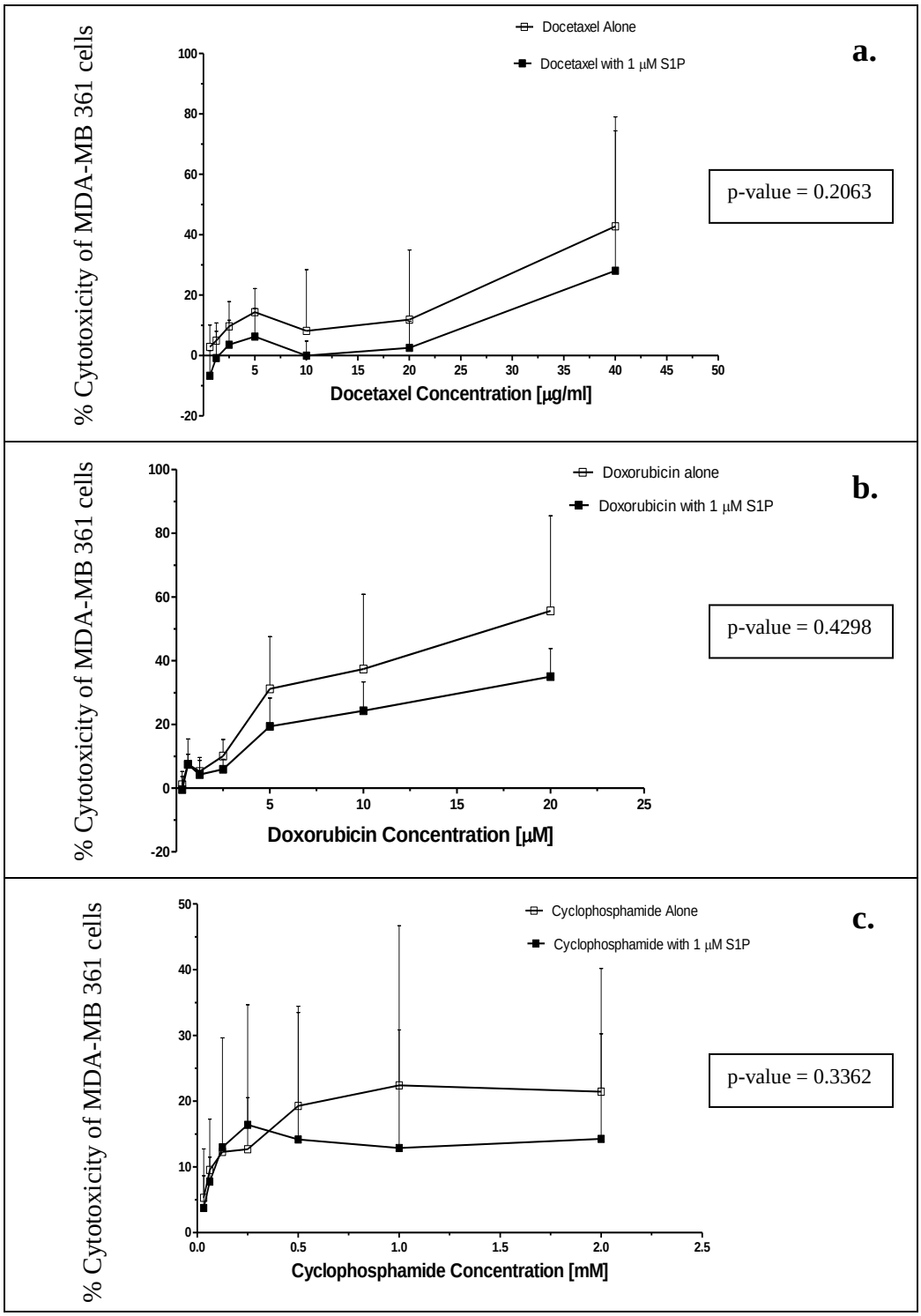


Figure 16. Percent cytotoxicity (mean ± SD) of the anticancer drugs against human breast cancer MDA-MB 361cells alone (▭) or in combination with 1 μM S1P (■), docetaxel (panel **a**), doxorubicin (panel **b**), and cyclophosphamide (panel **c)**. The cytotoxicity was measured 18 hours after the treatment. Cells treated with the complete growth media and the solvent were used as the control. The mean ± SD was calculated from three independent experiments.

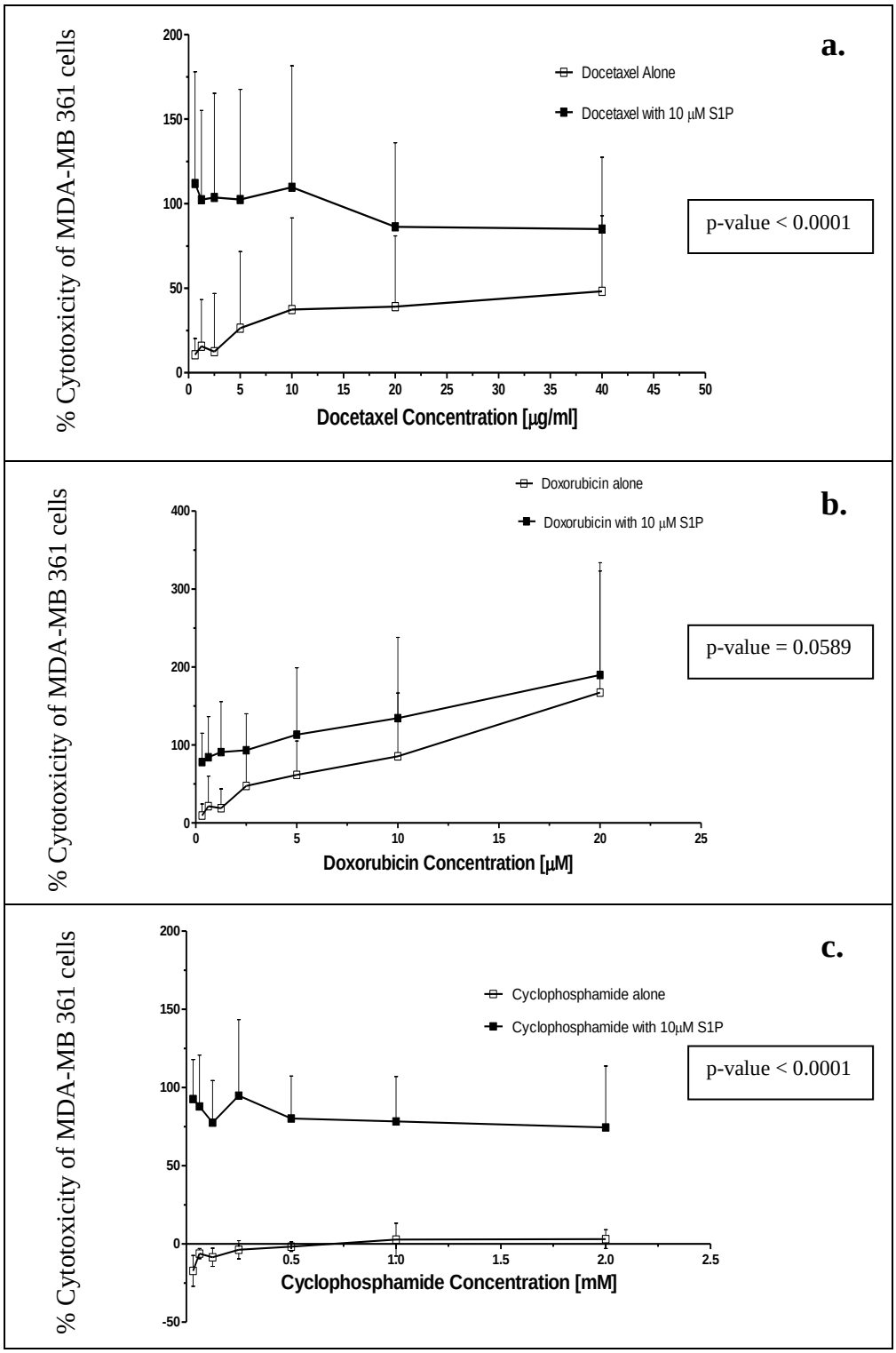


Figure 17. Percent cytotoxicity (mean ± SD) of the anticancer drugs against human breast cancer MDA-MB 361 cells alone (▭) or in combination with 10 μM S1P (■), docetaxel (panel **a**), doxorubicin (panel **b**), and cyclophosphamide (panel **c)**. The cytotoxicity was measured 18 hours after the treatment. Cells treated with the complete growth media and the solvent were used as the control. The mean ± SD was calculated from three independent experiments.

Table 4. Anticancer drugs IC_{50} ± SD values alone and with apoptotic and necrotic doses of sphingosine-1-phosphate.

Drug	IC_{50} ± SD*	
	MDA-MB 231 cells	MDA-MB 361 cells
Docetaxel alone	30.2 ± 34.1	17.8 ± 18.3
+ 1 µM S1P	1.2 ± 1.1	60.5 ± 63.1
+ 10 µM S1P	0.01 ± 0.01	0.3 ± 0.4
Doxorubicin alone	9 ± 16.8	24.5 ± 33.9
+ 1 µM S1P	1.3 ± 1.1	10.1 ± 8.8
+10 µM S1P	1.506000e+032 ± 0.00	92 ± 91
Cyclophosphamide alone	0.3 ± 0.4	1 ± 1.4
+ 1 µM S1P	0.04 ± 0.03	0.3 ± 0.4
+10 µM S1P	0.2 ± 0.3	0.02 ± 0.01

*IC_{50} shown for: Docetaxel is in µg/ml, Doxorubicin is in µM, and Cyclophosphamide is in mM
The cytotoxicity was determined by using Cytotox kit from Promega as described in materials and methods section. The IC_{50} was calculated by using GraphPad Prism software and mean ± SD of three independent experiments was used.

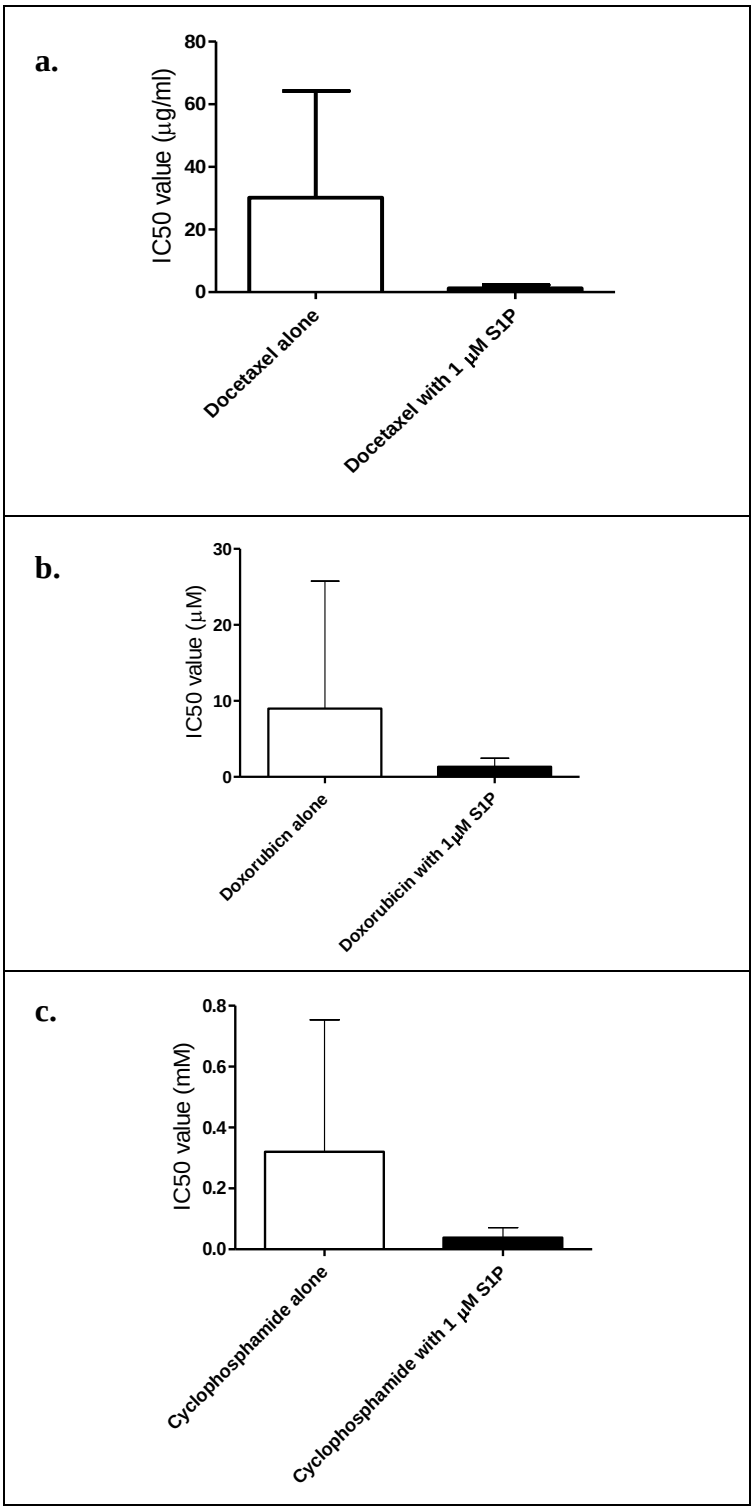


Figure 18. The IC_{50} (mean ± SD) of anticancer drugs against MDA-MB 231 breast cancer cells alone or in combination with 1 µM S1P, docetaxel (panel a), doxorubicin (panel b), and cyclophosphamide (panel c). The cytotoxicity was determined by using Cytotox kit from Promega as we describe in materials and methods. The IC_{50} was calculated by using GraphPad Prism software and mean ± SD of three independent experiments was used.

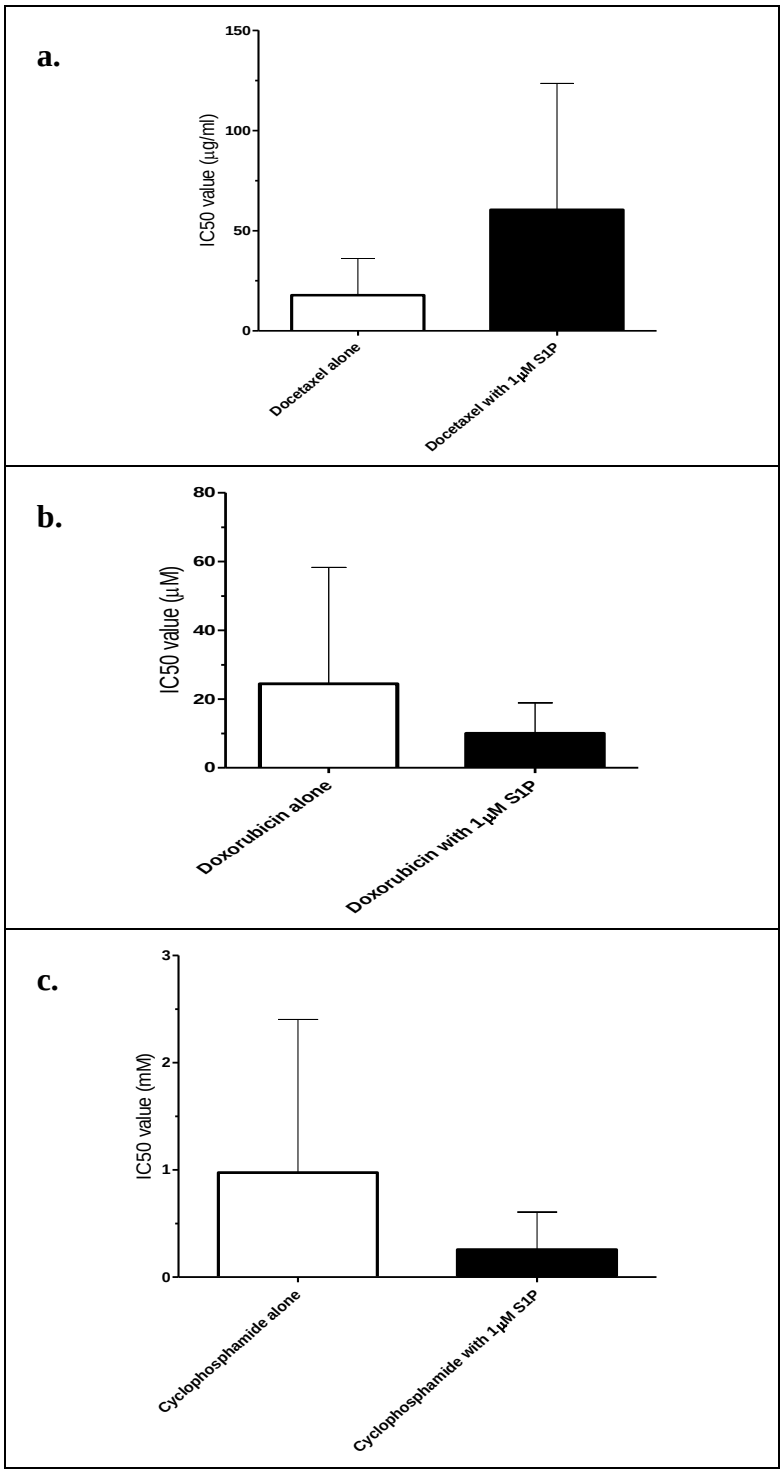


Figure 19. The IC_{50} (mean ± SD) of anticancer drugs against MDA-MB 361 breast cancer cells alone or in combination with 1 µM S1P, docetaxel (panel a), doxorubicin (panel b), and cyclophosphamide (panel c). The cytotoxicity was determined by using Cytotox kit from Promega as we describe in materials and methods. The IC_{50} was calculated by using GraphPad Prism software and mean ± SD of three independent experiments was used.

3.4. RAMAN IMAGING:

The S1P intensity was measured at point 1300 Raman shift/ cm^{-1} which is the strongest peak represent S1P. Figure 20 (panel a) indicates the presence of S1P inside the cells following exogenous administration.

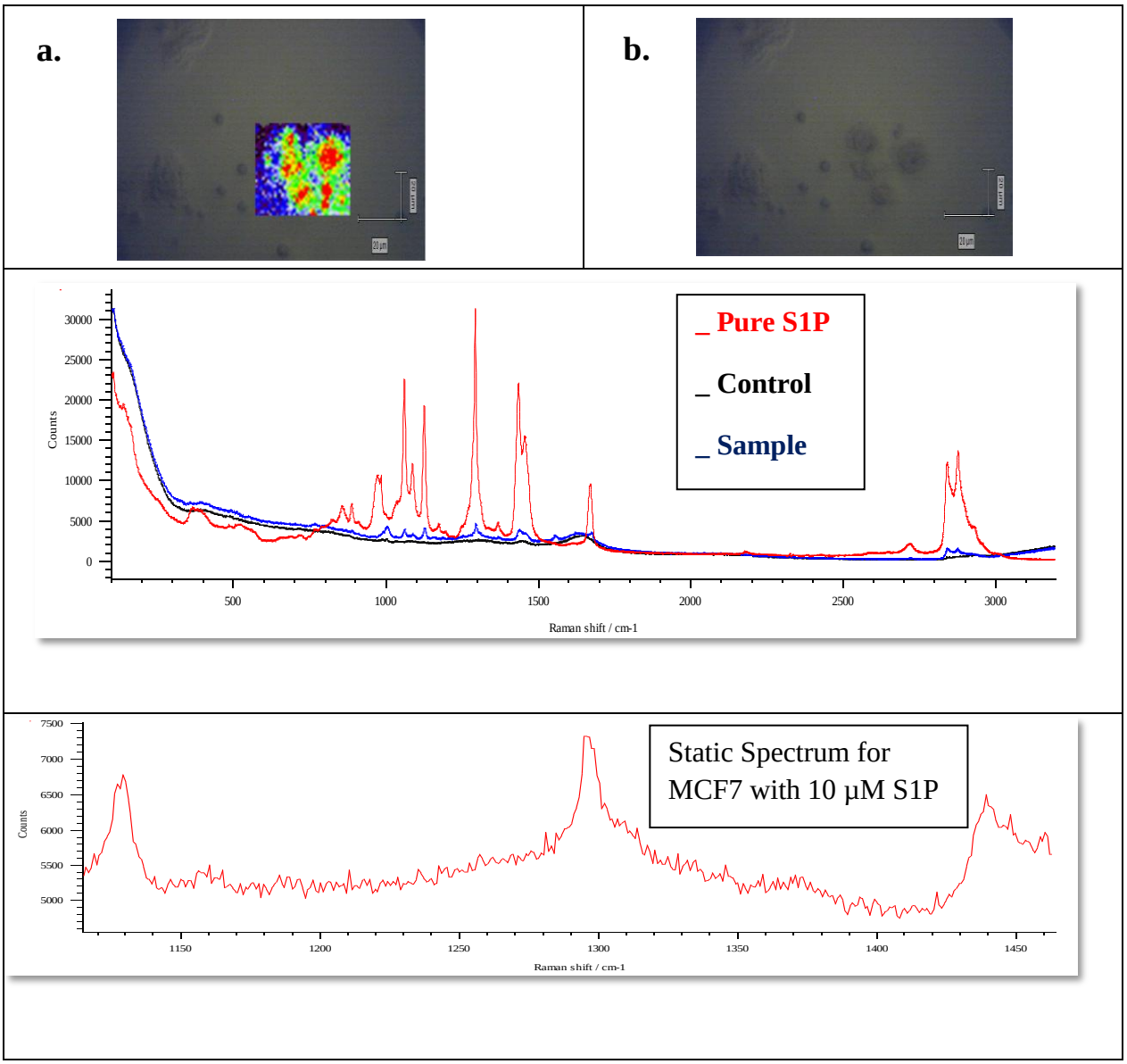


Figure 20. Raman Imaging data following the treatment of MCF7 cells with 10 µM S1P for 12 hr. Picture of the cells under the microscope (panel **b**), the confocal Raman image of the cells (panel **a**). The Extended spectrum of pure S1P [red line], control [black line] and experiment [blue line] (panel **c**). The static spectrum of the experiment (panel **d**).

3.5. ANIMAL STUDY:

During the experiment, I faced some difficulties in standardizing the tumour volume measurement method. Consequently, variations in the tumour volume were noted in the day -to-day measurement as shown in Figure 28 at the appendix page. In addition, the number of the nude mice that developed the tumour was low, the tumour induction success rate was around 46%, and the initial tumour size of the mice in each group was different. All of this contributes to an increase of the error bar of the results. However, similar experiment was done by our collaborator in China and showed that there was a reduction in the tumor volume in S1P, docetaxel and combination group compared to the control as shown in Figure 21 & 26, and the reduction was significant.

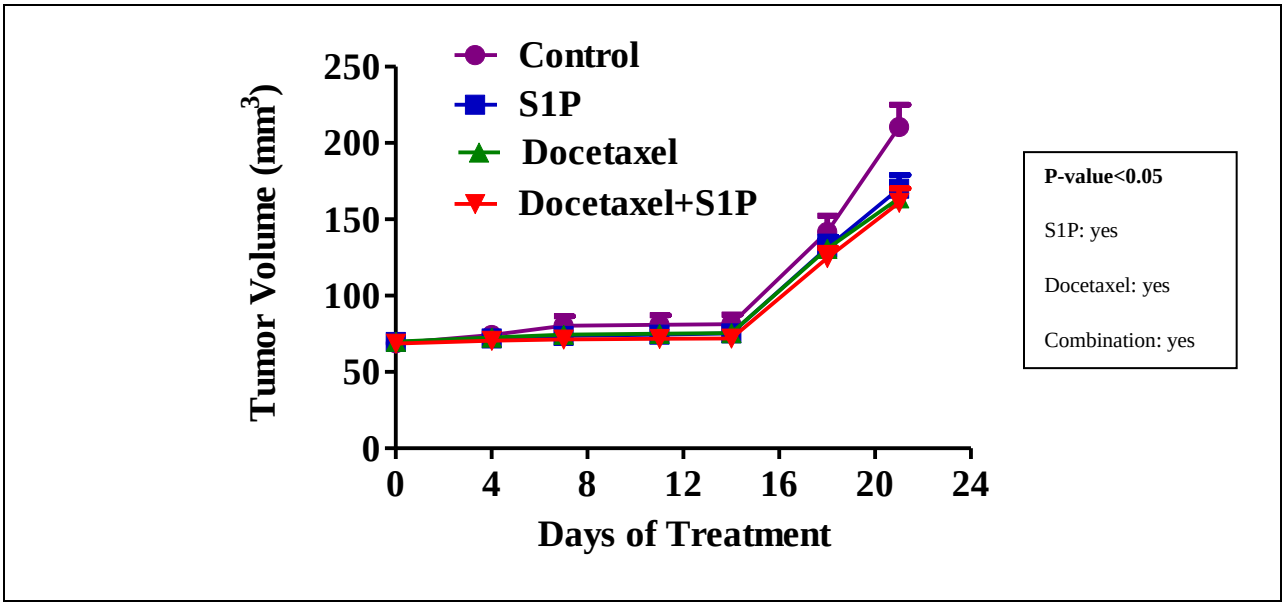


Figure 21. Animal study results. The tumour volume (mm^3) ± SD of the treated and control group during the 21 days of the experiment of MCF7 xenograft nude mice. In the last days of the treatment the tumour volume was different between the control group and the group who received the combination therapy. For the data analysis a One-Way ANOVA followed by Tukey's and Bonferroni's multiple comparison tests was performed by using GraphPad Prism.

3.6. IMMUNOHISTOCHEMISTRY STUDIES:

Histopathology was conducted by Dr. Bruce K. Wobeser (Assistant Professor, Anatomic Pathology and Oncology at the University of Saskatchewan).

3.6.1. HEART

In Figure 22, there was no sign of cardiac damage in the control and treated nude mice groups. In all of the sections there were neither morphologic alterations present nor any evidence of inflammation or necrosis.

3.6.2. LIVER:

In Figure 23, in the control and treated group, the liver tissue architectures were within normal limits and small numbers of neutrophils were observed within sinusoids. The hepatocytes of all the liver sections have mild anisocytosis and anisokaryosis with occasional binucleate cells. However, these changes were within the expected variability of mouse hepatocytes.

3.6.3. KIDNEY:

In Figure 24, the kidney tissue of the control and treated group show no sign of lesions. The architectures were within normal limits. There was no evidence of inflammation or necrosis. However, the tissue of one of the mice treated with docetaxel show exceptional small area of renal papilla where a small area of granular purple material was present. In size and shape it was similar to bacteria and there was no inflammation that was associated with it so it is likely of no significance.

3.6.4. TUMOUR

In Figure 25, in some of the sections there was no defined tumour mass present. There was abundant lymphocytic inflammation and fibroplasias scattered throughout the superficial dermis. However, in some of the treated group sections a single aggregate of tumour cells was found and

scattered lymphocytes were present within the neoplastic cells. No large or confluent areas of necrosis were present. Rare individual cells were shrunken and eosinophilic, which was consistent with apoptosis (Figure 25).

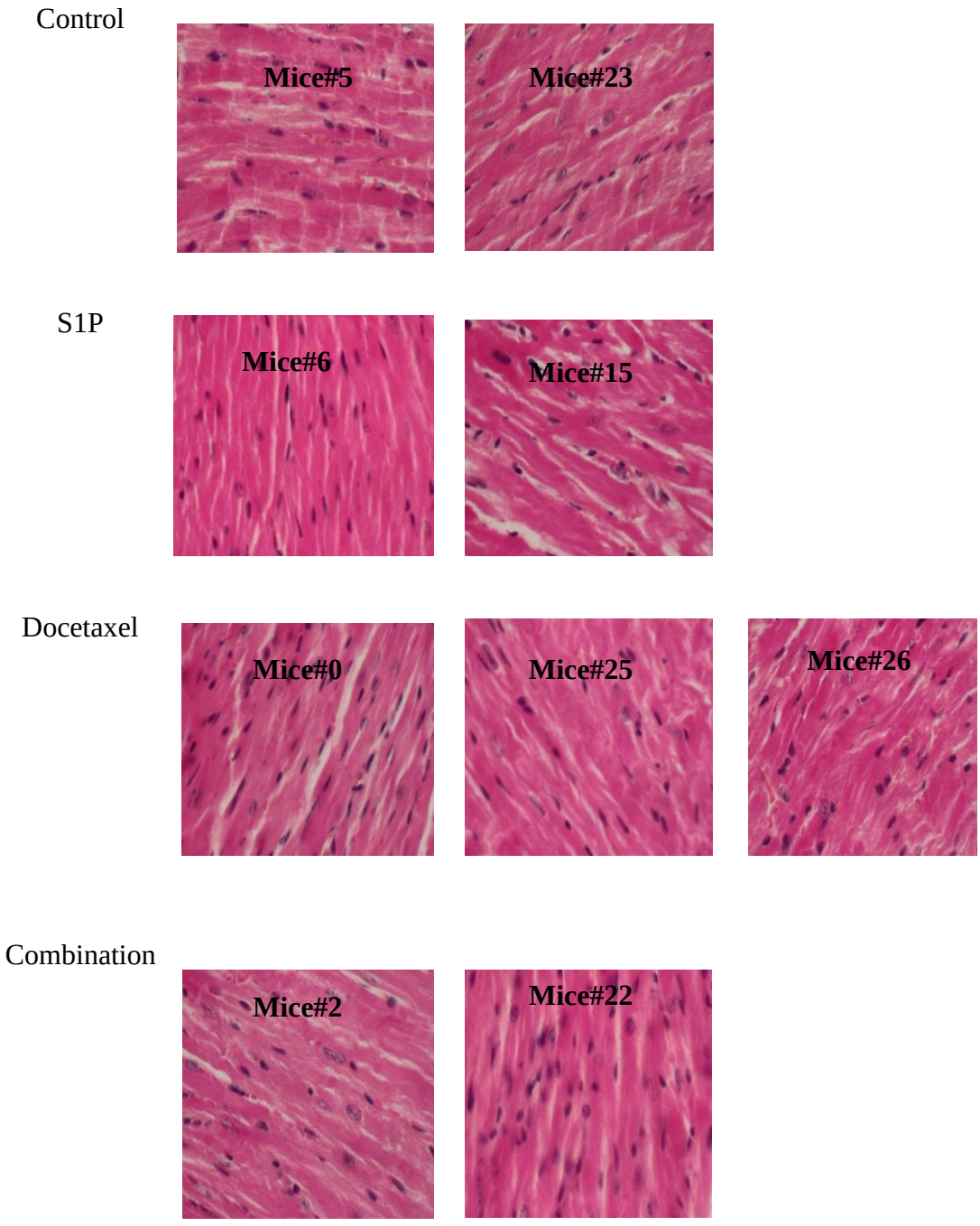


Figure 22. The immunohistochemistry of mouse heart tissue. Each slice is 5 mm in size and was stained with hematoxylin and eosin. No toxicity was associated with chronic *in vivo* exposure with intratumoural injections of sphingosine-1-phosphate. The microscope magnification view is 40x.

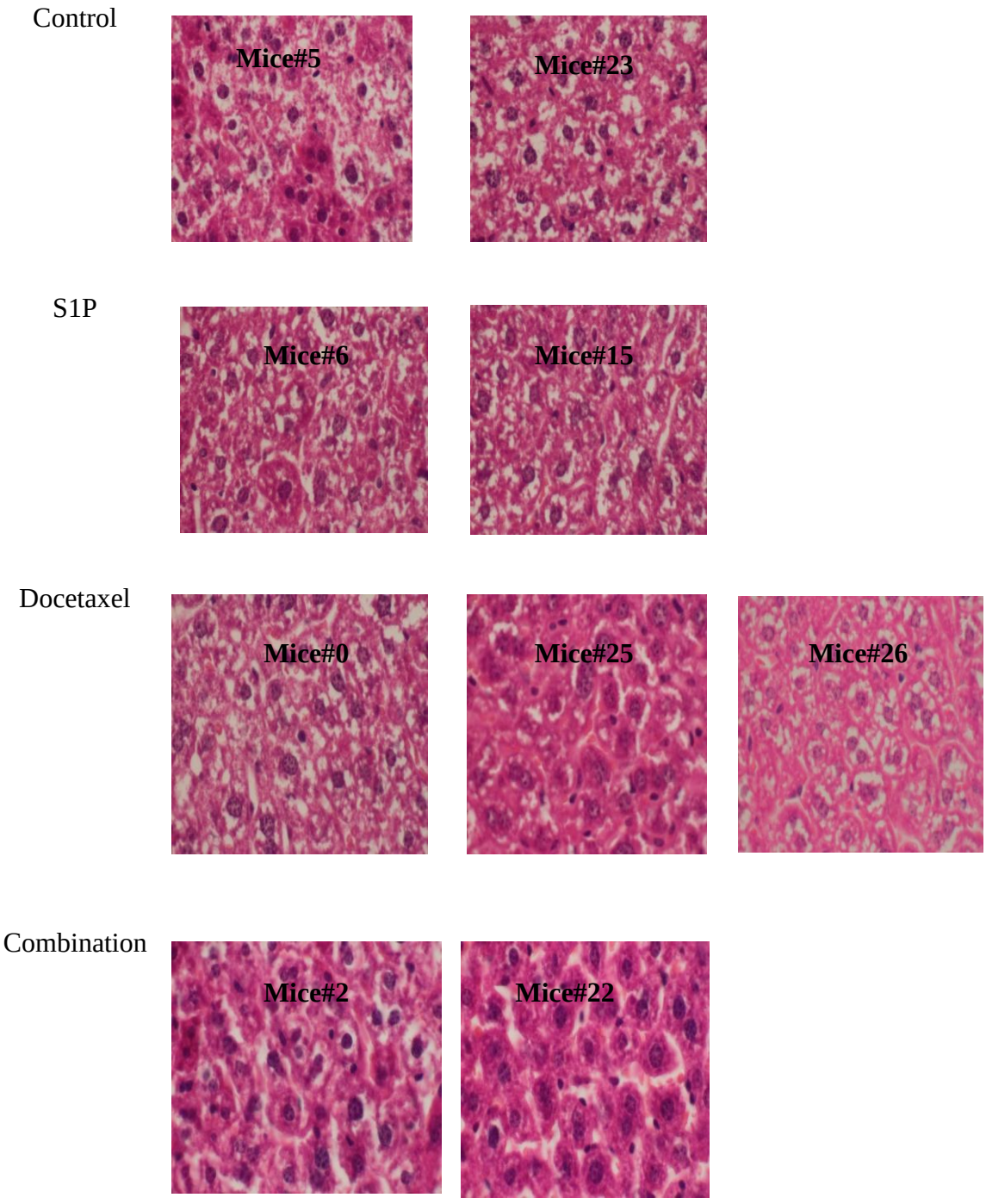


Figure 23. The immunohistochemistry of mouse liver tissue. Each slice is 5 mm in size and was stained with hematoxylin and eosin. No toxicity was associated with chronic *in vivo* exposure with intratumoural injections of sphingosine-1-phosphate. The microscope magnification view is 40x.

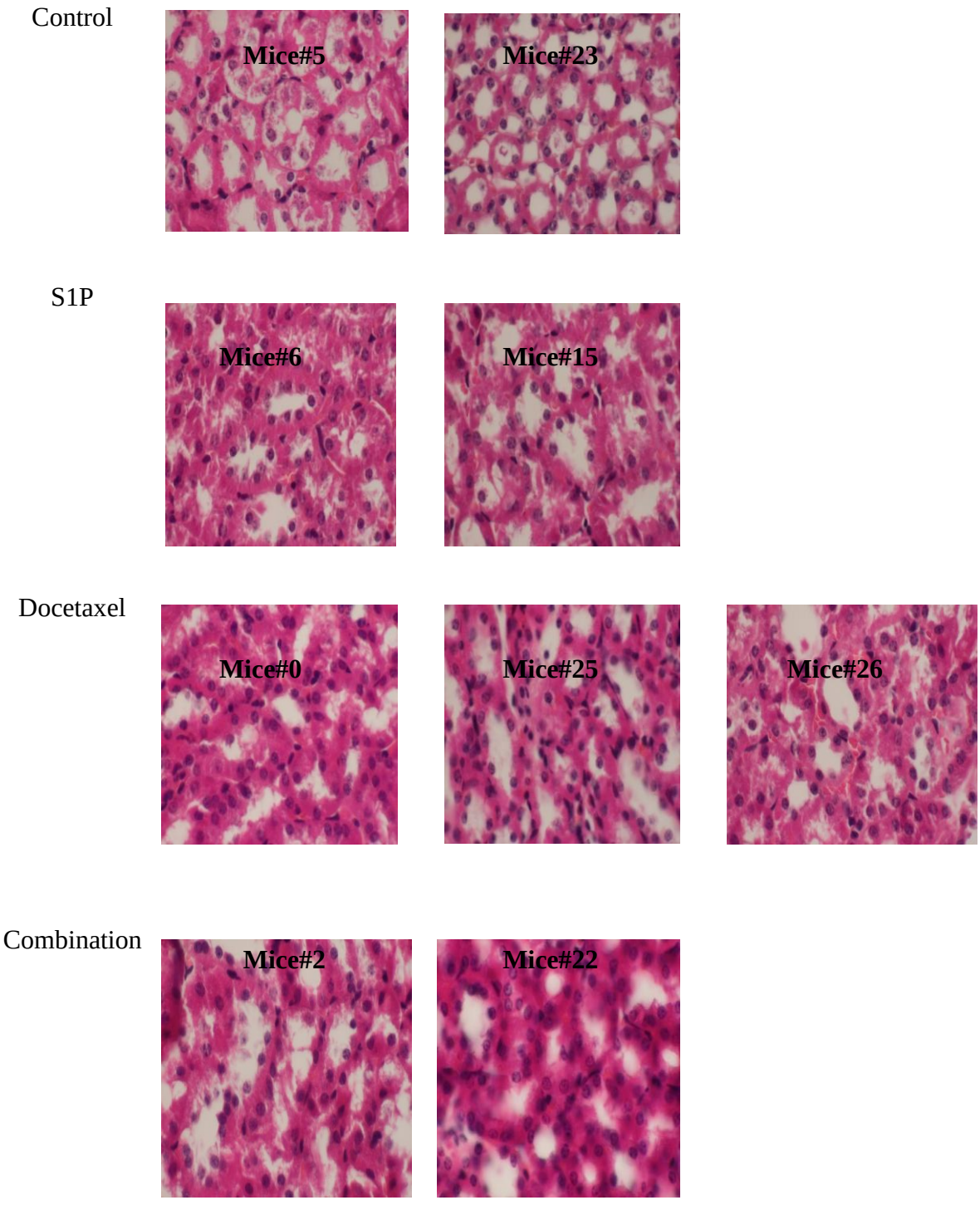


Figure 24. The immunohistochemistry of mouse kidney tissue. Each slice is 5 mm in size and was stained with hematoxylin and eosin. No toxicity was associated with chronic *in vivo* exposure with intratumoural injections of sphingosine-1-phosphate. The microscope magnification view is 40x.

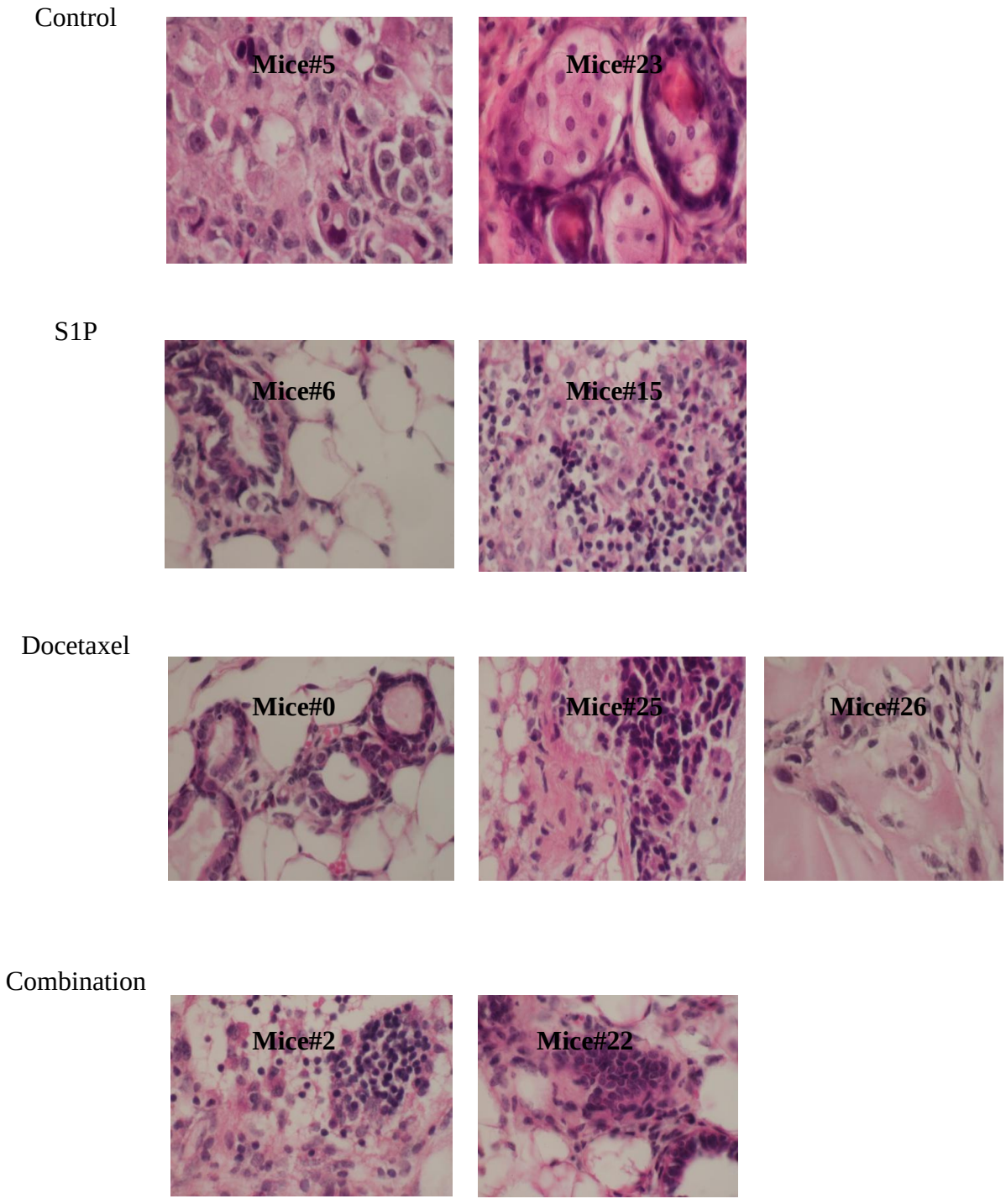


Figure 25. The immunohistochemistry of mouse tumour tissue. Each slice is 5 mm in size and was stained with hematoxylin and eosin. The microscope magnification view is 40x.

4. DISCUSSION

Natural biological compounds have been rich resources for medical applications in cancer treatment and prevention. The need to improve the cytotoxicity of the current available anticancer drugs continues to be important. Today there are a number of natural biological substances found to have the ability to induce apoptosis in different types of human tumour cells, such as sphingosine-1-phosphate (S1P). Several studies have demonstrated that S1P is associated with cell proliferation and survival potential making anti-S1P a good candidate for cancer treatment strategy. However, recent studies suggest the involvement of S1P in cell apoptosis (Oskouian & Saba, 2010; Davaille et al., 2000; Davaille et al., 2002; Moore et al., 1999). The exact function and role of S1P in normal and cancer cells need more clarification and this is exactly the goal of this research. I found that S1P is very similar to the newly indentified cytokine-induced apoptosis inhibitor, CIAPIN1, which has dual effects on cells cultures (Li, Wu & Fan, 2010). In my research I have done a number of experiments to evaluate the *in vitro* and *in vivo* effects of S1P against breast cancer cells.

4.1. SPHINGOSINE-1-PHOSPHATE IS MORE EFFECTIVE AGAINST AGGRESSIVE BREAST CANCER

In my research I obtained similar results to those reported by Spiegel et al. regarding S1P's effect on different types of breast cancer cells. S1P was more effective against aggressive breast cancer cells, such as MDA-MB 231 cells (Figure 9, 10, 11, 12 and 13). Aggressive breast cancer cells are usually associated with the absence of estrogen and/or human epidermal growth factor-2 receptors (i.e., ER-ve and/or HER-2 -ve). They are usually more invasive and possess higher metastatic potential. Aggressive breast cancer cells do not respond to hormonal therapy. Treatment of these cells with anti-estrogen, such as tamoxifen, is not effective and it is hard to find appropriate treatments to control their growth (Koduru et al., 2009). If the cancer cell

additionally lacks HER-2 receptors the treatment with monoclonal antibodies, such as trastuzumab, will fail. In addition, patients with ER-positive tumors have a median survival of 40 - 48 months, while patients with ER-negative have median survival of only 10 - 20 months (Koduru et al., 2009). In Spiegel et al.'s research they studied the effect of S1P at (1 or 10 μM) on the motility and proliferation of human breast cancer cells lines, including MCF7 and MDA-MB 231 cells. The MCF7 cells are ER-positive and HER-2 -ve, while the MDA-MB 231 cells are ER-negative and HER-2 -ve. Spiegel et al. found that S1P inhibited the motility and proliferation of both cell lines, but S1P effect was more potent against the MDA-MB 231 cells (Spiegel et al., 1994). In my research I examined the effect of S1P in other breast cancer cells including the MDA-MB 361 cells, which are ER-positive and HER-2 +ve, and compared its effects to the MDA-MB 231 cells. In the proliferation studies (Figure10) I found that treatment of these breast cancer cells with 10 μM S1P for 72 hr inhibited proliferation in both cell lines. However, in the MDA-MB 231 cells the percent inhibition of proliferation was 21%, whereas in the MDA-MB 361 cells the percent inhibition of proliferation was only 4%. Thus, the percent inhibition of proliferation was much higher in aggressive MDA-MB 231 cells. In my cytotoxicity studies, I have found that the IC_{50} in the MDA-MB 231 cells was 8.5 μM, while the IC_{50} in the MDA-MB 361 was 14 μM (Figure 12 and Table 3), suggesting S1P may kill the aggressive breast cancer cells at lower doses. Treatment of both cell lines with 10 μM S1P for 72 hr killed 39% of MDA-MB 231 cells and only 10% of MDA-MB 361 cells (Figure 13). S1P gave consistent response in the MDA-MB 231 cells compared with the MDA-MB 361 cells (Figure 12 and 13). In my present study, I found that S1P (at 1 and 10 μM) significantly enhanced the cytotoxicity of the chemotherapy drugs including docetaxel, cyclophosphamides, and doxorubicin against the MDA-MB 231 cells (Figure14 and 15). However, in the MDA-MB 361

cells only high concentration of S1P (i.e., 10 μM) enhanced the cytotoxicity of the chemotherapy drugs, while the low concentration of S1P (i.e., 1 μM) had no effect (Figure 16 and 17). From all of this, I conclude that S1P could be a useful agent in the treatment of aggressive and metastatic breast cancers, which are hard to treat by using current available treatment.

4.2. DIFFERENT EFFECT OF SPHINGOSINE-1-PHOSPHATE ON BREAST CANCER CELLS COMPARED WITH NORMAL BREAST CELLS

I have found that S1P induced apoptosis in the breast cancer cells at low concentrations without harming normal breast cells. The IC_{50} for apoptosis in the MDA-MB 231 breast cancer cells was 1.5 μM, whereas the IC_{50} in normal breast cells (i.e., MCF12A) was 10.1 μM (Figure 11 and Table 3). From the cytotoxicity studies I found that S1P caused necrosis in the MDA-MB 231cells with IC_{50} of 8.5 μM, and in normal MCF12A cells the IC_{50} was 13.2 (Figure 12 and Table 3). Thus, my results are consistent with Ling et al.'s research finding that S1P had different apoptosis response in MCF7 cells compared to MCF12A cells (Ling et al., 2011). They suggested that S1P could selectively induce apoptosis in the MCF7 cancer cells (Ling et al., 2011). My results showed that S1P induced apoptosis in breast cancer cells at low doses without harming normal cells. A major problem in current breast cancer therapies, such as chemotherapies, is that they lack the selectivity to tumors cells. Chemotherapies harm both normal and cancer cells leading to many undesirable effects that worsen the patient clinical outcome and increase the treatment cost. Chemotherapies are associated with nausea, vomiting, anemia, and neutropenia; because they kill rapidly divide cells, such as hair follicles, bone marrow, and gastrointestinal. To find an agent that is selective toward breast cancer cells will add a remarkable progress in improving the patient clinical outcome and will reduce the patients' suffering from the chemotherapies side effects. The S1P addition to breast cancer treatment may

reduce the breast cancer morbidity, improve the patient clinical outcome, and reduce the treatment cost.

4.3. SPHINGOSINE-1-PHOSPHATE IN COMBINATION WITH ANTI-CANCER DRUGS

In the present study, I found that S1P enhanced the effect of anti-cancer drugs, such as docetaxel, cyclophosphamide, and doxorubicin against ER+ve/HER-2 +ve and ER –ve/HER-2 -ve breast cancer cells *in vitro* (Table 4 and Figure 14, 15, 17, 18 and 19). These anti-cancer drugs are the most commonly used in breast cancer treatment (Chu & Sartorelli, 2009, p.900). Docetaxel is an antimitotic agent, which blocks cell division by inhibition of microtubule formation (Chu & Sartorelli, 2009, p.893). Cyclophosphamide is a nitrogen mustard alkylating agent, which arrests cell division by blocking the G2 stage of the cell cycle (Chu & Sartorelli, 2009, p.882-883). Doxorubicin is an anthracyclin antibiotic, which arrests cell replication by forming complex with DNA, and inhibition of topoisomerase II enzyme activity (Chu & Sartorelli, 2009, p.893-894). Although these drugs' mechanisms of action vary, we found that S1P can enhance their effectiveness regardless of the exact mechanism. However, there were slight differences in the percentage increase of cytotoxicity induced by S1P among these drugs. Also, in my study, I found that the percent increase of cytotoxicity of the anticancer drug was depended on the S1P concentration. The higher the S1P concentration the higher the cytotoxic effect can be achieved (Figures 14, 15, 16 and17). Co-administration of the anti-cancer drugs with high S1P concentration enhanced the cytotoxicity significantly (p-value < 0.0001) compared with low S1P concentration in MDA-MB 231 cells (Figure 14 and 15). Whereas, in the MDA-MB 361 cells low concentration of S1P did not enhance the cytotoxicity of anticancer drug, just the high concentration of S1P triggered the effect of the anti-cancer drugs (Figure 16 and 17). However, my findings are similar to the previous study conducted by Ling et al. that

exogenous administration of S1P can improved the docetaxel treatment potency in MCF7 breast cancer cells. The IC_{50} of docetaxel alone was 3.4 µg/ ml and its value from coadministration with 1 µM S1P was 2.1 µg/ ml (Ling et al., 2011). This means that S1P enhanced the cytotoxicity of docetaxel against MCF7 breast cancer cells. From my work, S1P was shown to enhance the cytotoxicity of anticancer drugs not only in MCF7 but in other type of breast cancer cells such as the MDA-MB 231 and MDA-MB 361 cells. Moreover, S1P can enhance the activity of diverse chemotherapy regardless of their mechanism of action. The S1P enhanced the cytotoxicity of anti-cancer drugs depending on two factors: the S1P concentration and the breast cancer type (i.e., ER+ve/ HER-2 +ve or ER-ve/ HER-2 –ve).

I undertook the *in vivo* studies of S1P alone or in combination with docetaxel in the BALB-C nude mice. Because of my lack of animal study experiences, my research results are not conclusive. Unquestionably, I faced some difficulties during the experiments as I mentioned previously in the results section. However, a similar study conducted through our collaborator in China showed that 1 µM S1P enhanced the cytotoxicity of docetaxel against MCF7 cells *in vivo* (Figure 21 and 26). The same collaborative group found similar results with the MDA-MB 231 xenograft nude mice model (Figure 27). However, these results indicated that MDA-MB-231 cells were more sensitive to S1P *in vivo* compared to MCF7 cells.

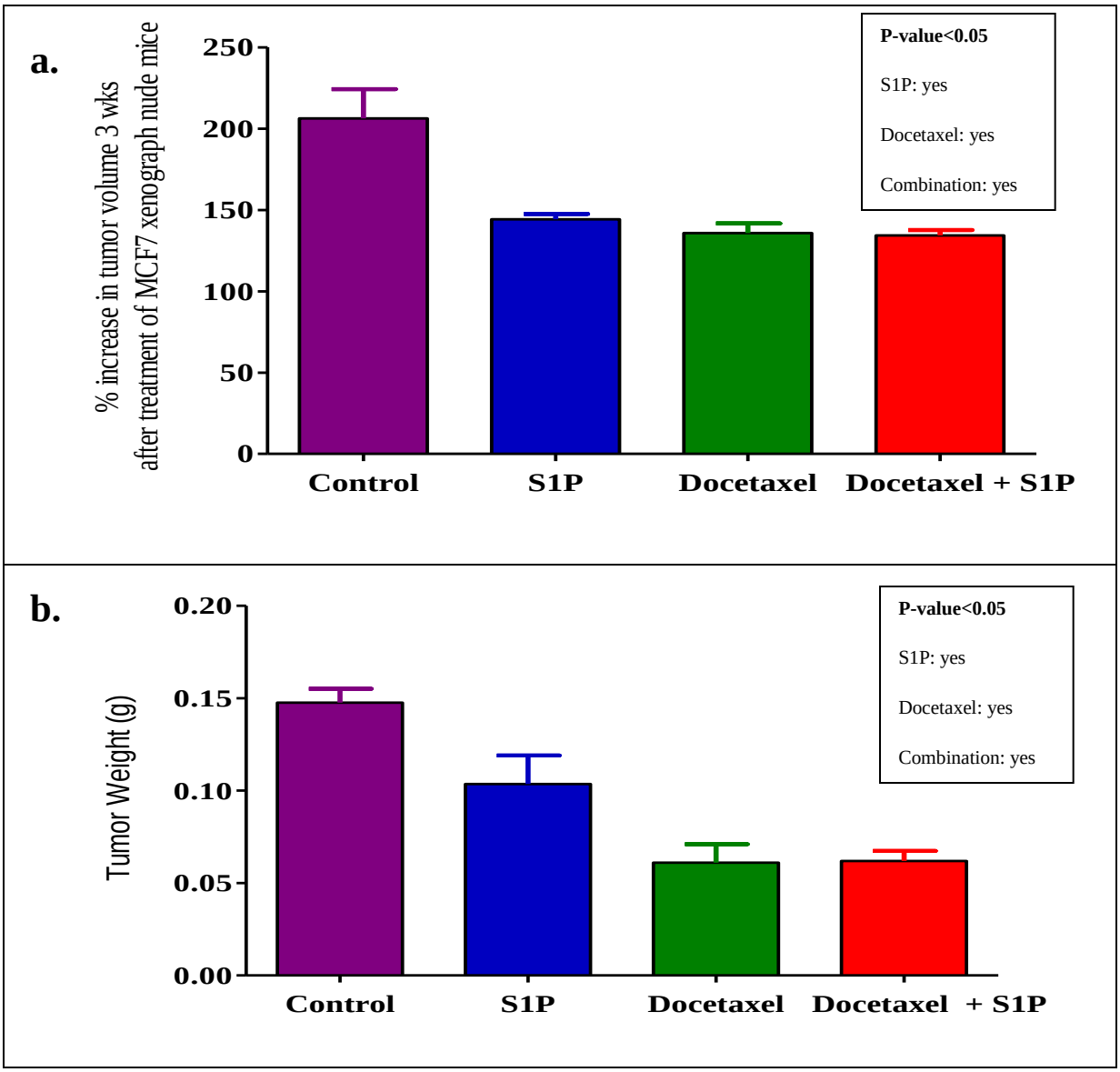


Figure 26. The percent increase of the tumor volume ± SEM of MCF7 xenograft mice after administration of docetaxel alone or in combination with sphingosine-1-phosphate (S1P) (**panel a**). The tumour weight mean (gram) ± SEM of MCF7 xenograft mice after administration of docetaxel alone or in combination with S1P (**panel b**). The mice received intra-tumoral injection and tumor dimension was measured daily for 21 days. The mean of each point was calculated from six nude mice.

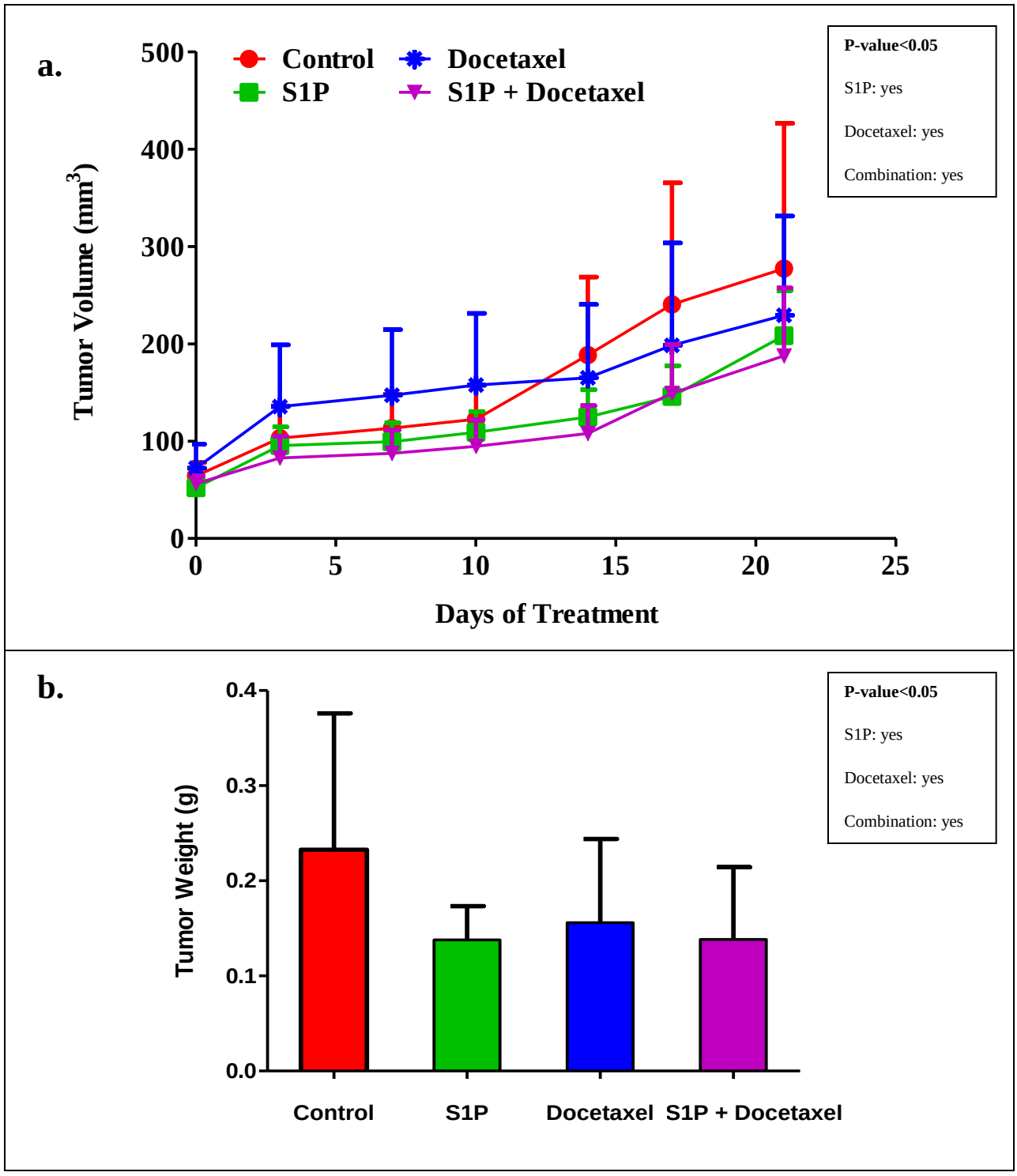


Figure 27. The tumour volume mean (mm^3) ± SD of MDA-MB 231 xenograft mice after administration of docetaxel alone or in combination with sphingosine-1-phosphate (S1P) (**panel a**). The tumour weight mean (gram) ± SEM of MDA-MB 231 xenograft mice after administration of docetaxel alone or in combination with S1P (**panel b**). The mice received intra-tumoral injection and tumor dimension was measured daily for 21 days. The mean of each point was calculated from six nude mice.

4.4. THE ANTI-PROLIFERATION EFFECT OF SPHINGOSINE-1-PHOSPHATE IS CORRELATED TO ITS INTRACELLULAR ACTION

In the present research, I studied the S1P cell distribution in MCF7 cells following exogenous administration of high concentration of S1P (i.e., 10 μM) by using the Raman imaging mapping. The literature revealed that external S1P will promote cell proliferation by activating S1PRs. However, my research suggests that external S1P undergoes a plasma-cell translocation (i.e., it can transport from the extracellular compartment to the cell) and that high concentration of S1P has anti-proliferation effects on breast cancer cells. I based this hypothesis on the fact that S1PRs are saturated at higher concentration of S1P and are only activated at nanomolar concentration (Davaille et al., 2000; Davaille et al., 2002). S1P can transport from plasma to the cell by ABCC7 transporter (Riboni, Giussani & Viani, 2010). From the Raman mapping I found that S1P was present mainly inside the cells following exogenous administration and its anti-proliferative effect was correlated to its intracellular action, Figure 20. This result is similar to previous studies including: (Wang et al, 1999), (Davaille et al, 2000; 2002), (Hung & Chuang, 1996), and (Moore et al., 1999). Wang et al., examined the involvement of S1PRs in the motility inhibition they observed following exogenous administration of S1P on the following breast cancer cell line: MCF7, ZR75-1, MDA-MB 231, and BT 549. They found that the S1P inhibition of cellular motility was independent of the receptors and it was associated with the intracellular action of S1P. Following the administration of S1P they measured its intracellular levels and found it was much higher than the control and this suggested that S1P inhibited cell motility by intracellular actions independent of the S1PRs (Wang et al., 1999). Davaille et al. examined the S1P proliferative effect on human hepatic myofibroblasts (hMF) and they found that S1P exhibited potent inhibition of hMF proliferation (IC_{50}= 1 μM). In this study they linked the anti-proliferative effect of S1P to its intracellular action and suggested the involvement of COX2.

Hung & Chuang found that S1P can induce apoptosis in human hepatoma cells (Hep3B) by increasing the expression of the Bax gene (Hung & Chuang, 1996). Moore et al. found that prolong exposure to 10 μM S1P induced apoptosis in hippocampal neurons by increasing the level of intracellular Ca^{2+} (Moore et al., 1999). All of these studies: (Wang et al, 1999), (Davaille et al, 2000; 2002), (Hung & Chuang, 1996), and (Moore et al., 1999) are consistent with our finding that the S1P anti-proliferative effect is associated with its intracellular action. However, we need to identify the S1P intracellular target that triggers its effect. More researches is needed to clarify S1P intracellular action.

4.5. DUAL EFFECT OF S1P ON BREAST CANCER CELLS

S1P has a dual effect on cell fate and migration. The basic function of S1P is still unclear. However, it was found that S1P concentrations determine its final effect. In my proliferation studies, I saw that S1P at low concentration enhanced cell proliferation but at higher concentration it exhibited an anti-proliferative effect (Figure 9). In those studies, I found that S1P exhibited its maximal proliferative effect at 1 μM and 2 μM in MDA-MB 361 and MDA-MB 231 breast cancer cells, respectively. S1P caused a stimulatory response at concentration lower than 2 μM and inhibitory response at a higher concentration in MDA-MB 231 cells and similar results were with MDA-MB 361 cells. My findings resemble other studies, such as the study conducted by Davaille et al and Moore et al. In Davaille et al.'s research they found that S1P has a dual opposing effect on hMF. It enhanced the cell proliferation of hMF by activation of S1PRs at nanomolar concentration and its apoptotic effect occurred at a micromolar concentration of S1P (Davaille et al., 2000). Moore et al. found that prolong exposure to 10 μM S1P induced apoptosis in hippocampal neurons. On the contrary, they found that prolong exposure to low levels of S1P (2 μM) did not cause any morphological change (Moore et al., 1999). To conclude,

in the present study I have demonstrated the opposite effects of S1P on two types of breast cancer cell the ER+ve / HER-2 +ve and ER-ve/ HER-2 –ve, suggesting, S1P exerts a dual effect on breast cancer cells.

4.6. CHRONIC EXPOSURE WITH INTRA-TUMOURAL INJECTIONS OF SPHINGOSINE-1-PHOSPHATE IS NON TOXIC

Although sphingosine-1-phosphate is an endogenous substance and less likely to cause toxicity *in vivo*, its effects when given exogenously should be examined. Especially, it is important to examine the effects of long-term exogenously administrated S1P on major organs, such as the heart, kidney, and liver. However, in my study there was no excessive systemic exposure of S1P, because I gave S1P as intra-tumoural local injection; redistribution of S1P to the systemic circulation dose occur, but systemic exposure levels are significantly lower than levels following oral or intravenous administration, administration routes which demand much higher doses of the compound to achieve pharmacological active levels at the site of action. In addition, we used low doses of S1P, i.e., 1 μM/dose, which is similar to the normal body level (0.2- 0.9 μM). In my immunohistochemistry studies we found that chronic exposure of S1P is less likely to cause harm to the heart, liver and kidney (Figures 22, 23, 24, and 25).

4.7. S1P INDUCED APOPTOSIS IN MDA-MB 361 BREAST CANCER CELLS BY CASPASE 3/7 INDEPENDENT PATTERN

In Figure 9 and 11, I found that S1P caused less proliferation and less apoptosis as S1P concentration increased in the MDA-MB 361 cells and these results oppose each other. The explanation in this variation of S1P response in the MDA-MB 361 cells is that S1P may induce apoptosis in the MDA-MB 361 cells by caspase 3/7 independent pattern. In this study I used Caspase-Glo® 3/7 assay to determine the percent increase of cell apoptosis induced by S1P in the MDA-MB 361 cells and the results shows that S1P caused less apoptosis. There are many

mechanisms by which cells undergo apoptosis and in the MDA-MB 361 cells S1P could induce apoptosis by a different mechanism compared to the MDA-MB 231 cells.

5. CONCLUSION

Despite the improvement in breast cancer patient clinical outcomes due to the use of the current available therapies, the morbidity and mortality rate of breast cancer remains high. There is a need to find new ways to treat breast cancer. In the last decade, technological enhancements have improved our understanding of breast cancer biology. Based on this knowledge new medications were developed to target different pathways, which correlate with breast cancer development. Targeting the apoptosis pathway is one of the new strategies developed to treat breast cancer and sphingolipids play a vital role in controlling both cell apoptosis and proliferation processes. Studying agents such as S1P that can act on both apoptosis and proliferation may offer a new treatment option in the current breast cancer drug arsenal. The main objectives of this study were to examine the role that S1P play in breast cancer and its potential application in breast cancer treatment. *In vitro* studies (i.e., apoptosis, proliferation, cytotoxicity, and raman imaging) and *in vivo* studies (i.e., the MCF7 xenograft nude mice model) were designed to determine whether S1P is a useful therapeutic agent and a good target for breast cancer treatment and prevention and whether S1P is a good candidate for combination therapy. Addition of S1P to chemotherapeutic drug regimens may decrease the toxicity, through anticancer drug dosage reduction and reduce the possibility of drug resistance development. The major findings of this study were that: S1P enhanced the cytotoxicity of anti-cancer drugs against breast cancer depending on the following factors: the type of the breast cancer cell and the concentration of S1P used. In addition, I found that S1P was more potent against aggressive breast cancer cell lines compared with other type of breast cancer cells. Moreover, I found that

S1P shows selectivity to breast cancer cells at low doses. Using a low dose of S1P induced apoptosis in breast cancer cells without harming normal breast cells.

6. FUTURE DIRECTIONS

The future directions of this research are many. The first direction, the effect of S1P on other type of breast cancer cells, particularly, triple negative breast cancer cells needs to be examined. This can be done by performing similar *in vitro* studies, including apoptosis, proliferation, and cytotoxicity studies, as performed in my thesis work. In addition, the apoptotic effect of S1P on MDA-MB 361 cells needs further evaluation. In my work I used Caspase-Glo®3/7 assay but we need to conduct a different assay such as the DNA Tunnel assay. The second direction is to study the expression of SIPRs on different breast cancer cells types. This can be done by extracting mRNAs for the S1PRs, especially, S1PR1, S1PR2 and S1PR3 from breast cancer cells. After that, Quantitative RT-PCR should be performed. These three receptors are known to be involved in cell fate and migration and assessing their expression in breast cancer cells is important to clarify S1P functions and role in breast cancer. The third direction is to study the effect of S1P on breast cancer motility. S1P is known to affect cell motility dependent on S1PRs expression in cells and it is involved in malignant cells progression and metastasis. This can be done by performing chemotaxis motility assay and from this test the S1P effect on different type of breast cancer cells motility can be examined. The fourth direction is to evaluate the expression of the three enzymes that determine the S1P level, which are S1P-phosphatase, S1P-lyase and SK, on different type of breast cancer cells. The expressions of these enzymes determine the S1P effect on breast cancer cells. This can be done by extracting the mRNAs for these enzymes from breast cancer cells then performing Quantitative RT-PCR. The fifth direction is to elucidate the S1P intracellular target that triggers its apoptotic effect. My thesis work suggests that S1P induces

apoptosis and inhibits proliferation of breast cancer cells. However, the mechanism by which S1P induces apoptosis requires investigation. This can be done by performing Quantitative RT-PCR to examine the effect of S1P on different apoptosis pathways. Any alteration on the expression of the genes involved in the apoptosis may identify the mechanism. The expression of p21, p53, Bax and Bcl-2 may provide some answers. Once we detect the gene that is associated with S1P effect we can silence the gene and examine the impact on breast cancer cells. In addition, other intracellular functions of S1P need more clarification. The sixth direction is to uncover S1P's role in the mechanism of drug resistance development. Some studies suggest the interaction of S1P with an efflux transporter, such as P-glycoprotein. S1P's effect on P-gp needs further evaluation to clarify S1P role in drug resistance development in tumours. Over-expression of P-gp and other efflux transporters in tumour cells is a major obstacle in cancer treatment and understanding S1P's role may solve this major problem. This can be done by performing Quantitative RT-PCR to examine the effect of S1P on the expression of P-gp in different type of human breast cancer cells. The seventh direction is to find the causes of potent and consistence effect of S1P in aggressive breast cancer cells as compared with less aggressive breast cancers cells. Does the estrogen receptor or human epidermal growth factor expression interfere with S1P overall effect on different type of breast cancer cell? It is known that estradiol stimulate SK1 and increase the release of S1P through activation of ABCC1 and ABCG2 transporters (Pyne & Pyne, 2010). The final direction, we need to examine the S1P effect *in vivo* alone or in combination with anti-cancer drugs. This can be done by evaluating S1P on different types of breast cancer xenograft nude mice models. For example, we could develop a triple negative breast cancer xenograft nude mice model and examine the effect of S1P alone or in combination with anti-cancer drugs, such as docetaxel, cyclophosphamide, or doxorubicin.

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8. APPENDIX

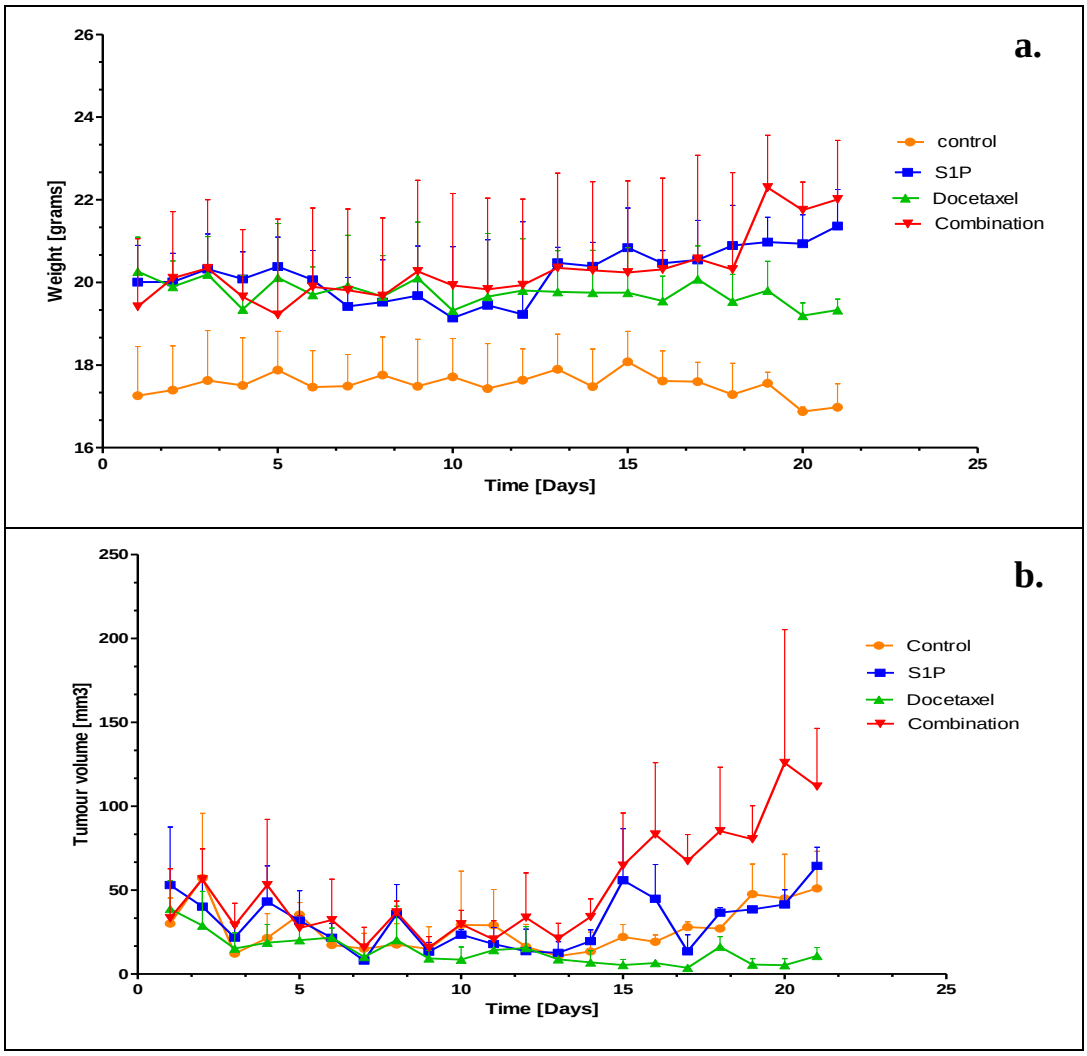

Figure 28. Animal study results. The mean weight of the mice (gm) ± SD of treated and control groups during the whole experiment **(panel a)**. The tumour volume (mm^3) ± SD of the treated and control group during the 21 days of the experiment **(panel b)**. The tumour volume is significantly different between the control group and the group who received the combination therapy, while there was no significant difference between the control and the other group. For the data analysis a One-Way ANOVA followed by Tukey's and Bonferroni's multiple comparison tests was performed by using GraphPad Prism.